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**House Dust Mite Induced Gene Expression and Cytokine
Secretion by Human Dermal Fibroblasts**

A thesis submitted in partial fulfillment of the requirements for the degree
of Master of Science

Jananie Rockwood

BS. Biological Sciences, University of Cincinnati, 2009

2012

Wright State University

WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

July 18, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Jananie Rockwood, ENTITLED House Dust Mite Induced
Gene Expression and Cytokine Secretion by Human Dermal Fibroblasts BE
ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
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Abstract

Rockwood, Jananie. M.S., Department of Biological Sciences, Wright State University, 2012. House Dust Mite Induced Gene Expression and Cytokine Secretion by Human Dermal Fibroblasts.

House dust mites are a group of mites that are commonly found in homes worldwide. The three most commonly found species of house dust mites in temperate environments in the United States are *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Euroglyphus maynei*. House dust mite allergens are strongly associated with asthma, perennial rhinitis and atopic dermatitis. Whole dust mite extracts contain both allergen and non-allergen molecules that can influence cell functions in the skin, mucous membranes of the nose and eyes, and epithelial linings of the lungs. Extracts from the three different house dust mite species contain different molecules that induce different responses. The purpose of this study was to investigate how molecules in house dust mite extracts modulate IL-6, IL-8, MCP-1 and M-CSF cytokine gene expression and secretion by normal human dermal fibroblast (NHDF) cells. This study demonstrated that in some instances cytokine gene expression and secretion were induced while in other instances only stored cytokine was released by NHDF cells.

Table 1 List of Abbreviations

Abbreviation	Definition
DF	<i>Dermatophagoides farinae</i>
DP	<i>Dermatophagoides pteronyssinus</i>
EM	<i>Euroglyphus maynei</i>
EU/ml	Endotoxin Unit/ ml
HDM	House Dust Mite
IL-6	Interleukin-6
IL-8	Interleukin-8
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemotactic Protein-1
M-CSF	Macrophage Colony Stimulating Factor
NHDF cells	Normal Human Dermal Fibroblast cells
PARs	Protease Activated Receptors
PmB	Polymyxin B
TLRs	Toll-like Receptors

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I. Introduction

A. Biology of House Dust Mites

1. Important Species

Mites belong to the subphylum Chelicerata of the phylum Arthropoda. Chelicerae are appendages that can vary in form and function. Dust mite chelicerae contain both moveable and fixed digits that are pincer-like which helps distinguish them from other members of this subphylum (Arlian and Platts-Mills, 2001). Mites are physiologically and morphologically different from insects. They are arachnids so they are more closely related to spiders and scorpions than insects (Arlian and Platts-Mills, 2001). Like other arachnids, mites have four pairs of legs.

Mites are ubiquitous and exploit many different microenvironments including the nasal passages of birds and mammals and the skin and hair follicles of mammals. Many of these species are parasites of plants and mammals. Some parasitic species suck blood or fluid from their animal or plant host.

House dust mites are a group of mites that are found in homes and are known to induce an allergic reaction in individuals that can lead to allergic rhinitis, asthma and atopic dermatitis. The three most commonly found house dust mite species in the United

States are *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Euroglyphus maynei*. These house dust mite species are the source of multiple allergens. House dust mites are found in places of human contact, especially homes and in lesser density in public access places including schools and work places (Arlian et al., 2002). Within homes, they are found predominantly in areas where shed skin scales collect and serve as their main source of food. Areas where high concentrations of skin scales collect include: sofas, carpets and mattresses. Their water source is ambient water vapor, thus a humid environment favors their prevalence. Thriving populations can be supported in cracks and crevices of tiled and hardwood floors where their food and moisture from the air collect. Dust mite allergens are a component of dust and disperse to areas like curtains and bedding (Arlian et al., 2002).

2. Geographic Prevalence and Variation

Globally house dust mites are found in humid environments as they obtain water from humid, ambient air. However, there is tremendous variation between the population densities of dust mites between and within geographic locations (Arlian et al., 2002). Longitudinal field studies have shown that mite densities fluctuate seasonally in parallel with outdoor relative humidity. Indoor relative humidity usually fluctuates in parallel with outdoor relative humidity. In temperate climates, mite densities are higher during

more humid summer months than during the dry winter. The low mite density in winter is due to the fact that heating within homes reduce the relative humidity (Arlian et al., 2001).

In the United States, both *D. farinae* and *D. pteronyssinus* are commonly found together in homes in temperate and humid environments (Arlian et al., 2002). However, it has also been observed that within a home, there is a higher population of one species over the other (Arlian, 1992). *E. maynei* is also found in temperate environments. Its ecology and distribution has been studied less intensively when compared to the studies done on *D. farinae* and *D. pteronyssinus*. However, in some homes in the United States *E. maynei* may be the dominant species (Arlian et al., 2002).

3. Role of Relative Humidity and Water Balance

Water is essential for the survival of all terrestrial animals including dust mites. House dust mite bodies have a large surface-to-volume ratio, which would increase the rate of water evaporation. Water loss could also occur through processes like excretion, reproduction and feeding. Adaptations that reduce water loss as well as to increase the intake of water other than by drinking are crucial in dust mite species survival. One such adaptation is a tough exoskeleton that prevents water from evaporating from their

bodies. Dust mites also lack an organized respiratory system, which also reduces the loss of water vapor (Arlian, 1992).

Dust mites gain small amounts of water through ingestion of moist food, imbibition (if water is freely available), as a product of the oxidation of organic molecules and through passive absorption of moisture in unsaturated air (Arlian, 1992). However, the main source of water for house dust mites is through active absorption of water vapor from ambient air when relative humidity is sufficiently high (Arlian, 1977). Thus, a relatively humid (51% and greater) environment increases the species prevalence (Arlian et al., 2002).

The relative humidity of the environment has been shown to influence mite intake of food. Dust mites' food source is relatively low in water content. The moisture in the air equilibrates with the mites' food source. This means that the moisture content of the food ingested is proportional to the relative humidity of its environment. A study done on the feeding of *D. farinae* and *D. pteronyssinus* (Arlian, 1977), showed that at 85% relative humidity, mites consumed a daily quantity of food that was 42% greater than their body weight. At 75% relative humidity, the daily consumption of food was significantly reduced and was only 8.4% for *D. farinae* and 10.3% for *D. pteronyssinus* greater than their body weight. Therefore, controlling relative humidity may have an

effect on the buildup of mite allergens in homes. Fecal matter is a source of dust mite allergens. Fecal accumulation has been shown to be high when the feeding rate of mites is high. Therefore, lowering the relative humidity of an environment would ensure a slower feeding rate and a low accumulation of allergens found in fecal matter (Arlian, 1992).

4. Reproduction and Life Cycle of Dust Mites

The life cycle of dust mites consists of the following stages: eggs, larva, protonymph, tritonymph and adult. Molting and changes in morphology separate each of the developmental stages. The larval and nymphal stages have an active feeding period followed by a quiescent period (inactive) prior to the molt to the next life stage (Hart, 1998).

Larvae: The eggs develop into larvae. Mite larvae have 3 pairs of legs and no genitalia or reproductive organs. The larvae have some dorsal and lateral body bristles or setae. Ventral and genital setae are absent at this stage (Arlian, 1989).

Protonymphs: This stage has one pair of genital papillae (nipple-like structures). Dorsal, ventral and anal setae are also present. Protonymphs are octapodal (4 pairs of legs) (Colloff, 1992).

Tritonymphs: This stage is octapodal, and has two pairs of genital papillae. In addition to dorsal, ventral and anal setae, tritonymphs also have middle and anterior genital setae (Colloff, 1992).

Adults: Males and females have genital systems and openings and are larger than the developing nymphs. Adult mites are sexually dimorphic. Female mites are also larger than male mites. The female mites are distinguished by the presence of a bursa copulatrix, seminal receptacle, and duct complex at the posterior end. Male mites have anal suckers (Colloff, 1992).

House dust mite reproduction involves exclusive mating between male and females. Males attach to the female mite by anal suckers, which orientates their bodies in opposite directions. The sperm is transferred into the female via the bursa copulatrix. The female is mobile during copulation, which can take up to 48 hours. During which time the male, still attached to the female is dragged along (Hart, 1998).

D. farinae takes 35.6 ± 4.4 days to complete its life cycle at 23°C and 75% relative humidity. At these conditions female mites survive a total of 100 days (Arlian and Dippold, 1996). Female mites are able to reproduce for 34 of these days and a single female has the ability to lay 0-5 eggs per day.

D. pteronyssinus takes 34 ± 5.9 days to complete its life cycle at a temperature of 23°C and 75% relative humidity. The female mites can survive for 31 days under these growth conditions. During this period, female mites are able to reproduce for 26.2 days and lay 0 to 8 eggs per day (Arlian et al., 1990).

The optimum reproductive and growth conditions for *E. maynei* have been studied less extensively. The developmental cycle of this species can take 25-33 days to develop from egg to adult at a temperature of 25°C and 75% relative humidity (Taylor, 1975; Hart and Fain, 1988).

B. Medical Importance

1. Prevalence of Allergy to Dust Mites

Dust mite allergens are strongly associated with asthma, perennial rhinitis and atopic dermatitis (Arlian and Platts-Mills, 2001). Due to the reactions dust mite allergens can induce, understanding how these molecules cause an allergic response is essential.

Whole mite extracts are used in immunotherapy and skin testing. Atopic dermatitis is considered to be a complex disease and therefore a causal immunotherapy to treat this disease does not exist. However, there are several approaches that are used to treat it. For example one approach for treating atopic dermatitis is the use of specific

immunotherapy (SIT). SIT inhibits IgE mediated allergic diseases. A study conducted by Werfel et al. (2005) showed the positive effect that immunotherapy like SIT has on atopic dermatitis patients. Patients who had atopic dermatitis and were sensitized to house dust mite extracts showed improved eczema following SIT containing house dust mite extract.

It is likely that mite extracts used for medical purposes contain both non-allergenic and allergenic molecules (Arlian et al., 2008). Therefore, it would be beneficial to identify how mite extracts influence the inflammatory response as well as understanding its effects on normal cell functions. Understanding how mite extracts influence cell function would also be essential in developing target specific immunotherapies and skin testing drugs.

C. Allergens

1. Characterization, Biochemistry and Nomenclature of Allergens

Allergens are antigens that cause an allergic reaction or hypersensitivity in individuals. Mite allergens are associated with mite body and fecal matter includes enzymes involved in the molting and digestion processes, and secretions such as saliva (Arlian

and Platts-Mills, 2001, Thomas et al., 2010). More than 21 different allergens have been identified (Table 1) (Thomas et al., 2010, Arlian, 2002).

Allergens are named using a particular strategy where the first three letters of the genus name is followed by a space, the first letter of the species name, which is followed by the group number (represented as an Arabic numeral) (Arlian, 2002). No part of the allergen's name is italicized. If the first few letters of the genus and species are the same then an additional letter should be added to either the genus or species.

Initially characterized allergens were assigned a group number based on the chronological order of discovery. Subsequent allergens that shared similar properties were then added to the already identified groups. Allergens that have >67% amino acid similarity, similar biochemical functions and structure are assigned to the same group. The allergen nomenclature only consists of those molecules that have greater than 5% IgE reactivity. Major allergens are those that induce IgE reactivity in greater than 50% of the individuals tested (Arlian, 2002). Group 1 and 2 allergens are major contributors to dust mite extract allergenicity. This is based on IgE binding. Groups 4, 5, 7 and 21 are other important contributors to allergenicity of dust mite extract (Thomas et al., 2010).

Table 2: Biochemical properties of known house dust mite allergens.

Allergen Group	Biochemical Properties	MW (kDa)
Group 1	Cysteine protease	25
Group 2	Lipid binding protein	14
Group 3	Trypsin-like serine protease	28-30
Group 4	Amylase	56-63
Group 5	Unknown	14-15
Group 6	Chymotrypsin-like serine protease	25
Group 7	Lipid-binding protein	22-28
Group 8	Glutathione-S-transferase (rat and mouse)	25-26
Group 9	Collagenolytic serine protease	24-28
Group 10	Tropomyosin	33-37

* References: Thomas et al., 2010, Arlian 2002)

Group 1 allergens are the best-characterized dust mite allergens. They have been identified as cysteine proteases. An extensively studied group 1 allergen is Der p 1 that was isolated from *D. pteronyssinus* fecal matter. Der f 1, isolated from *D. farinae* is another group 1 allergen and it shares 80% sequence homology with Der p1. Therefore, cross reactivity is known to occur between Der p 1 and Der f 1 but these allergens also contain species-specific epitopes (Arlian, 2002).

Group 2 allergens also have been extensively studied. These allergens are lipid-binding proteins associated with secretions from male mite's reproductive tracts or proteins associated with molting. Der p 2 and Der f 2 show 88% sequence homology. They are cross-reactive and also have species-specific epitopes (Thomas et al., 2010, Arlian, 2002).

2. The allergic reaction

Allergic reactions are induced when allergic individuals come into contact with allergens. Allergenic molecules do not affect non-allergic individuals. Type 1 hypersensitivity (allergic reaction) involves immunoglobulin E (IgE) antibodies. Allergic asthma, rhinitis and atopic dermatitis, food allergies and some insect bites are all associated with type 1 hypersensitivity (Platts-Mills, 2001). A runny nose, red or puffy eyes, wheezing, itching and swelling are the most common symptoms (hay fever or atopy) of the allergic reaction.

Vascular permeability and chronic inflammation are some ways in which dust mite allergens can affect patients with allergic asthma (Platts-Mills, 2001). The group 1 house dust mite allergen Der p 1 in addition to affecting epidermal cells in the lungs has also been shown to enhance allergenicity by increasing IgE antibody specificity (Gough et al.,

1999). Another group 1 allergen Der f 1 has been shown to affect eosinophils. A study by Miike and Kita (2003) showed that Der f 1 influences eosinophils to release granular proteins and thus increase the symptoms an allergic individual experiences.

The allergic reaction in genetically pre-disposed individuals involves the sensitization to allergens and the activation of T-helper 2 (Th₂) cells and B lymphocytes (B cells). Antigens that enter the body are internalized, processed and incorporated into major histocompatibility II (MHC II) molecules by antigen presenting cells (APCs) such as macrophages, B cells and dendritic cells. APCs then present the MHC II bound antigens to immature T cells (naïve T cells). T cells that recognize MHC II molecules are activated (CD4⁺ T cells) and differentiate into Th₂ cells. The sensitization phase of an immediate hypersensitive reaction involves the release of cytokines such as interleukin-4, 5 and 13 (IL-4, IL-5 and IL-13) by Th₂ cells. The cytokine IL-4 binds to IL-4 receptors on B cells, which causes isotype switching of its immunoglobulin class to immunoglobulin E (IgE). B cells differentiate into plasma cells and secrete IgE antibody. The constant region of secreted IgE, known as Fcε3, binds to high affinity receptors (FcεRI) on mast cells and basophils or low affinity CD23 receptors (FcεRII) on eosinophils and B lymphocytes (Presta et al., 1994). IgE bound cells are then established on mucosal linings and the skin. Cytokines IL-4 and IL-5 are also involved in regulating eosinophil functions and

influence the growth of mucosal-type mast cells. Cytokines such as IL-4 and IL-13 enhance IgE production (Miike and Kita, 2003).

Subsequent exposure to the allergen activates the IgE-bound mast cells on mucosal linings and the skin. The allergen binds to the variable region of the IgE antibodies. Cross-linking occurs when an allergen binds to the variable regions of two adjacent antibodies (Bradding et al., 2002). Cross-linking and the uptake of calcium ions cause degranulation. This triggers the release of proinflammatory mediators such as histamines, tryptase, leukotrienes and prostaglandins. These mediators are responsible for the immediate (within minutes) allergic response (Bradding et al., 2002; Borish et al., 1999). The mediators produced by mast cells also cause a delayed inflammatory reaction that occurs 4 to 8 hours after the immediate reaction (Bradding et al., 1992; Borish et al., 1999).

D. How Molecules in Mite Extracts Affect Cells

1. Lung Epithelial Cells

House dust mite allergens inhaled has been shown to affect the function of epithelial cells of the lungs. In patients who have allergic asthma, these allergens can cause bronchial constriction and the release of cytokines and chemokines that cause an

inflammatory response in the lungs. Studies have shown that house dust mite allergens that exhibit cysteine and serine protease activity (i.e. group 1 and 3 allergens respectively) increase permeability in the lungs by breaking down tight junctions between epithelial cells. The increased permeability between epithelial cells contributes to the 'leakiness' of lung airways (Winton et al., 1998; Herbert et al., 1995, Stewart et al., 1994).

A study by Kauffman et al. (2006) showed that the protease Der p 1 caused morphological changes in lung cells and desquamation. Damage to lung cells would result in an increased amount of allergen movement across mucosal membranes, which triggers the release of interleukin 6 and 8 (IL-6 and IL-8) by epithelial cells (Kauffman et al., 2006; Asokanathan et al., 2002).

Allergens that exhibit protease activity like those that belong to group 1 and 3 act through protease-activated receptors (PARs) (Thomas et al., 2010, Kauffman et al., 2006 and Asokanathan et al., 2002). PARs are 7-transmembrane G-protein coupled receptors that are expressed on many cell types including the endothelial cells of blood vessels, connective tissue, leukocytes and epithelial cells. Stimulation of PARs results in the mobilization of intracellular free Ca^{2+} . Der p 1 has been shown to function through the PAR 2 specific pathway

Lung epithelial cells can also be affected by allergens such as Der p 5 that do not have specific protease activity. Kauffman et al. (2006) showed that Der p 5 can affect epithelial cell morphology and induce the release of IL-6 and IL-8. This study showed that Der p 5 mobilizes intracellular Ca^{2+} through a calcium dependent pathway that does not use PARs. The authors speculate that the pathway through which Der p 5 operates involves the use of toll-like receptors (TLRs).

TLRs are proteins that play a key role in innate immunity. The TLR family consists of membrane spanning non-catalytic receptors that recognize microbial components like endotoxins and other pathogen-associated molecules. TLR-2 and TLR-4 are two receptors that recognize the endotoxin lipopolysaccharide (LPS) to trigger an immune response (Hammad et al., 2009, Cardoso et al., 2007, Takeda and Akira, 2005 and, Gangloff and Guenounou, 2004). TLR-2 and TLR-4 have also been shown to act through a pathway that requires the adaptor protein MYD88. The LPS molecule is bound to the TLR with the aid of MYD88 to trigger the inflammation process (Takeda and Akira, 2005).

House dust mite extracts that have been shown to affect lung epithelial and airway dendritic cells act through pathways that involve TLR-2 and TLR-4 (Hammad et al., 2009, Kauffman et al., 2006, Asokanthan et al., 2002). For instance *D. pteronyssinus* allergen Der p 2 has been shown to prompt an immune response through TLR-4 (Trompette et

al., 2009, Hammad et al., 2009). Like the group 2 allergens, Mueller et al. (2009) showed that Der p 7 is able to interact with TLRs to promote an innate immune response.

2. Skin Cells (Keratinocytes, Fibroblasts and Endothelial Cells)

The skin is the body's first barrier to infection and when damaged or broken can lead to the entry of bacteria and allergens. The skin is exposed to the environment and it can be damaged by factors such as exogenous chemicals, ultraviolet radiation, protease activity and mechanical injury.

The epidermal layer of the skin contains corneodesmosomes which are made up of adhesion proteins that provide structural integrity. Protease activity on the dermal barrier affects the functioning of corneodesmosomes by causing desquamation. Desquamation is a process by which corneodesmosomes are cleaved by protease activity. As a result of desquamation, the epidermal barrier is broken allowing the entry of foreign molecules like allergens and skin irritants (Vasilopoulos et al., 2007).

Atopic dermatitis is a chronic inflammatory skin disease that is triggered by allergic stimulants like dust mite material (Vasilopoulos et al., 2007, Cork et al., 2006). Atopic dermatitis can either be extrinsic or intrinsic. Intrinsic atopic dermatitis does not involve

IgE mediated sensitization. Extrinsic atopic dermatitis is characterized by IgE mediated sensitization. A study by Novak et al. (2003) showed that 70-80% of patients exhibited the extrinsic atopic dermatitis and 20-30% exhibited the intrinsic form of atopic dermatitis. Deleuran et al. (1998) show that Der p 1 and Der p 2 proteolytic activity causes irritation when patch testing is conducted on patients with atopic dermatitis.

At a cellular level, whole house dust mite extracts have been shown to affect the function of specific cells of the skin. Arlian et al. (2008) show that whole dust mite extracts influence normal human epidermal keratinocytes in a dose dependent manner. Normal human epidermal keratinocytes that were sensitized with dust mite extract were tested for the up or down secretion of interleukin 1 receptor antagonist (IL-1ra), growth related oncogene α (GRO- α) and transforming growth factor α (TGF- α) and compared to media and LPS (lipopolysaccharide) controls. When the cells were treated with *D. farinae* and *D. pteronyssinus* extract, there was no change in secretion of IL-1ra and TGF- α when compared to the media and LPS controls. The keratinocytes showed increased levels of production of GRO- α showed high levels of production when the cells were treated with low concentrations of these two mite extracts but there was no significant change in secretion when treated with a higher concentration of mite extract. Keratinocytes treated with a low concentration of *E. maynei* extract showed low levels

of secretion of IL-1ra and TGF- α but, high levels of GRO- α when compared to the media and LPS controls. High doses of *E. maynei* extract sensitization resulted in no change of GRO- α production and an increased amount of IL-1ra secretion when compared to the media and LPS controls.

Arlan et al. (2008) also showed that normal human dermal fibroblast cells that were stimulated with the dust mite extracts would up or down regulate secretion of IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1) and macrophage colony stimulating factor (M-CSF) compared to media and LPS (lipopolysaccharide) controls. Stimulation with *D. farinae* extract resulted in secretion of increased levels of IL-6, IL-8, MCP-1 and M-CSF when compared to the media control. The secretion of M-CSF was low when compared to the LPS control. Stimulation with *D. pteronyssinus* extract resulted in high levels of IL-8 and M-CSF secretion and no change to the levels of IL-6 and MCP-1 when compared to the media control. However, when the secretion of these cytokines was compared to the LPS control, IL-6 and MCP-1 showed lower secretion levels and there was no significant difference to the production of IL-8 and M-CSF. Stimulation with *E. maynei* induced an increased amount of IL-8 secretion and no change in the production of IL-6, MCP-1 and M-CSF when compared to the media control. The production of IL-8

remained unchanged whereas the production levels of IL-6 and MCP-1 were lower when compared to the LPS control.

Mite extracts have also been shown to influence the regular functions of normal human dermal micro vascular endothelial cells (Arlian et al., 2009). Whole mite extracts of *D. farinae*, *D. pteronyssinus* and *E. maynei* were used to sensitize the endothelial cells in the presence and absence of polymyxin B (PmB). PmB is an antibiotic that acts as an endotoxin inhibitor by binding to lipid A. Lipid A is the major component of endotoxin. In the absence of PmB, intracellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1) and E selectin expression was stimulated by all three mite extracts. IL-6, IL-8, MCP-1 and GM-CSF were also secreted as a response to dust mite stimulation. Interestingly endothelial cells that were sensitized with *E. maynei* extract that had inhibited endotoxin activity did not show a change in the cell surface molecules expressed. IL-8 and MCP-1 were also secreted at lower levels. This means that molecules other than endotoxins in whole mite extracts influence endothelial cell functions. All three mite extracts in the presence of PmB secreted constitutive amounts of IL-6, G-CSF and GM-CSF. Therefore, this study showed that even when endotoxin activity in mite extract was prevented, normal human dermal microvascular cell functions are still affected by mite extracts.

E. Research Objectives

House dust mite allergens are strongly associated with asthma, perennial rhinitis and atopic dermatitis. Whole mite extracts are used in immunotherapy and skin testing. The mite extracts used contain molecules that are both allergenic and non-allergenic. These molecules can influence cell functions of the skin, mucus membranes of the nose and eyes, and the epithelial lining of the lungs. Thus, it would be beneficial to identify how molecules from mite extracts can influence allergic and inflammatory reactions. Understanding how whole mite extracts influence skin cell function would also be essential in developing specific immunotherapies and aqueous extracts.

Previous research in the lab has shown that whole dust mite extracts influence the secretion of cytokines by normal human dermal fibroblasts and keratinocytes. The purpose of this study is to determine if the molecules found in mite extracts modulate the gene expression as well as secretion of cytokines in normal dermal fibroblasts.

II. Methods

A. Overview

House dust mite extracts were used to challenge cultured normal human dermal fibroblast (NHDF) cells. Challenging the cells with mite extracts was essential in understanding how house dust mite extracts affect NHDF cells of the skin. The studies were conducted in three phases as follows:

The house dust mite extracts used were *D. farinae*, *D. pteronyssinus* and *E. maynei*. After the cells were challenged with 50 µg/ml of house dust mite extracts for 6, 12 and 24 hours, the supernatants from the cell cultures were collected and the cells were harvested. Gene expression studies were conducted on the harvested cells. Gene expression of interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) was evaluated to determine if there was a change in the expression of these genes over time. Cytokine-specific enzyme-linked immunosorbent assays (ELISA) were carried out on the cell culture supernatants collected after a given period of stimulation to determine the amount of cytokine secretion for IL-6, IL-8, MCP-1 and M-CSF.

In a separate set of experiments, NHDF cells were also challenged with mite extracts for shorter time periods (1, 6 and 12 hours) with lower doses of mite extract. Again, the supernatants were collected after the period of stimulation and ELISAs for the four cytokines of interest were carried out to determine if low doses and shorter time intervals affected cytokine secretion. Gene expression was not determined for these short stimulation time experiments.

Hose dust mite extracts contain endotoxins (LPS), proteases and many other types of molecules (Arlian et al., 2008). LPS stimulates cells through Toll-Like Receptors (TLRs) and proteases act through Protease Activated Receptors (PARs). Normal human dermal fibroblast cells were challenged with modified extracts in order to ascertain the possible mechanisms through which molecules in house dust mite extracts work.

To evaluate the ability of house dust mite extracts to affect NHDF cells through a pathway involving TLRs, the extracts were treated with polymyxin B to inhibit LPS in the mite extract. The ability of the mite extract to influence cytokine secretion was then determined by stimulating the NHDF cells with the treated extracts.

To assess the ability of house dust mite extracts to affect NHDF cells through a pathway involving PARs, the extracts were boiled to denature the enzyme or treated with

immobilized trypsin to denature proteins. SDS PAGE was used to visualize any change in the protein profile of the extracts. The ability of the modified mite extracts to influence cytokine secretion was then determined by stimulating the NHDF cells with the treated extracts for 6 hours.

B. Detailed Protocols

1. Tissue Cultures

Adult normal human dermal fibroblast (NHDF) cells were acquired as cryo-preserved aliquots from Cambrex Bio Science Walkersville Inc. (Walkersville, MD., USA). The cells were maintained at 37°C and 6% CO₂. Supplier recommended Fibroblast Growth Medium 2 (FGM-2) supplemented with fibroblast growth factor, insulin, gentamicin sulfate, amphotericin B and bovine serum was used as growth medium.

A frozen aliquot of cells was allowed to thaw and added to 12 ml of FGM-2 growth medium. Cells were grown in BD Falcon 75 cm² tissue culture flasks (Bedford, MA., USA) until confluent. The NHDF cells were then split according to general laboratory procedures and allowed to reach 60-70% confluence before being challenged with house dust mite extracts. All studies were performed on cells that were between

passages 4 to 11. The media and solutions involved in maintaining and harvesting the cells were Lonza manufactured products distributed by Fisher Scientific (Pittsburgh, PA).

2. Preparation of Whole Mite Extract

D. farinae, *D. pteronyssinus* and *E. maynei* whole dust mite extracts were prepared from pure mite bodies harvested from thriving cultures. Approximately 1.2-1.3 g of pure mite bodies for each mite was added to approximately 11 ml of endotoxin free water (LAL H₂O). The mixture was refrigerated at 4°C overnight and the next day homogenized using a Ten Broeck homogenizer. Insoluble particles from the homogenates such as mite exoskeletons, mite bodies and fecal matter were removed by centrifugation. The supernatant contained water soluble molecules. The supernatant from each mite extract mixture was then filter sterilized (0.22 µm) into 5 ml vial and stored at 4°C (Yadav et al., 2006).

The protein concentration for each house dust mite extract was determined using Bradford Protein Assay as previously described (Arlian et al., 2009). Briefly, the standard used to quantify protein concentration was Bovine Serum Albumin (BSA). The Bradford Protein Assay was set up by first carrying out a 1:20 dilution of 1.47 mg/ml of BSA in 12 X 75 glass tubes. A doubling dilution in bullet tubes for *D. farinae*, *D. pteronyssinus* and

E. maynei whole dust mite extracts was also set up. The tubes were vortexed and the contents of each tube were transferred to 12 X 75 glass tubes.

Protein assay dye purchased from Bio-Rad Laboratories Inc. (Hercules, CA) was added to the tubes of diluted whole dust mite extracts and BSA standard. The tubes were vortexed and the contents of each tube were transferred to disposable cuvettes.

A spectrophotometer was used to read absorbance values at a wavelength of 595 nm. The spectrophotometer was zeroed and re-blanked before reading the cuvettes. The 1:20 BSA standard tubes' absorbance readings were first established before absorbance readings were determined for diluted house dust mite extracts. The protein concentration for each mite extract was then computed using the absorbance readings.

3. Challenging Human Fibroblast Skin Cells with Whole Dust Mite Extract

The whole house dust mite extracts (50 µg/ml) was used to challenge NHDF cells in the presence or absence of 50 ng/ml of *Escherichia coli* lipopolysaccharide (LPS). LPS is a major component of the outer membrane of gram negative bacteria. LPS is an endotoxin and is known to activate an inflammatory response by acting through Toll Like Receptors (TLRs) and would serve as a positive control in challenging the cells. The cells were also stimulated with boiled extracts (50 µg/ml) in order to determine if

denaturing heat labile proteins found in the extracts affects cytokine gene expression and secretion. The extracts used for this purpose were boiled for 30 minutes.

In order to determine if the period of stimulation with whole dust mite extract affects cytokine gene expression, two trials were set up. The first trial (Trial 1) consisted of two different experiments; the first was stimulation with dust mite extract for 6 and 24 hours. The second trial (Trial 2) was a time course experiment where NHDF cells were challenged for 6, 12 or 24 hours.

After the normal human dermal fibroblast cells were challenged for the desired time intervals, the supernatants from the 25 cm² tissue culture flasks were aspirated and frozen in 5 ml eppendorf tubes in order to perform cytokine-specific enzyme-linked immunosorbent assays (ELISA). The cells were then harvested and used for cytokine gene expression studies.

4. Gene Expression

Gene expression assays were conducted to determine qualitative gene expression levels for cytokines IL-6, IL-8, MCP-1 and M-CSF. These assays were also essential in determining if cytokine gene expression were time sensitive.

Reverse transcription was carried out on cell lysates that were generated from 1×10^5 harvested normal human dermal fibroblast (NHDF) cells. This was essential for creating cDNA templates needed for quantitative PCR (qPCR). IL-6, IL-8, MCP-1 and M-CSF gene expression was detected using FAM dye during qPCR.

A more detailed method of the relevant steps in gene expression is described as follows.

i. Cell Lysis

NHDF cells were harvested after a given time period of stimulation (e.g.: 6, 12 or 24 hours). 1×10^5 cells were pelleted in eppendorf tubes and placed on ice. The cells were resuspended in cell lysis buffer and incubated for 10 minutes at 75°C. The cells were placed back on ice after incubation and 2 µl of DNase I was added to each tube. The mixture was mixed gently and incubated at 37°C for 20 minutes to degrade the genomic DNA. In order to inactivate DNase I, the mixture was incubated at 75°C for an additional 5 minutes.

ii. Reverse Transcription

Eppendorf tubes containing 5 µl of cell lysates were placed on ice. A mixture consisting of the following supplier recommended reagents: 4 µl of dNTP mix, 2 µl of random decamers were added to each tube. The tubes gently vortexed and heated for 3

minutes at 70°C to denature any secondary DNA structures that could impeded enzyme activity. The tubes were placed on ice and 2 µl of reverse transcription buffer, 1 µl of reverse transcriptase and 1 µl of RNase inhibitor was added to each one. The tubes are incubated at 42°C for 1 hour. The temperature is raised to 95°C for 10 minutes to inactivate the reverse transcriptase. The cDNA preps were stored at -20°C.

iii. Quantitative Polymerase Chain Reaction (qPCR)

For each cytokine being tested (IL-6, IL-8, MCP-1 and M-CSF), a PCR mixture was made. The PCR mixture which contained the following supplier recommended reagents: 1 µl of 20x Taqman gene expression assay, 10 µl of 2x Taqman gene expression master mix and 6 µl of RNase-free water. The PCR mixture was added to eppendorf tubes containing 3 µl of cDNA. Each cDNA template was tested in triplicates. The tubes were centrifuged briefly to ensure complete mixing. An optical 96 well reaction plate was loaded with the cDNA-PCR mixture and sealed with a clear PCR adhesive film.

The qPCR and qualitative analysis was carried using SDS 2.2.2 software which was compatible with the PCR machine CGR ABI 7900 HT SDS in the genomic research center at Wright State University.

Cells to cDNA kits were purchased from Applied Biosystems (Foster City, CA., USA) for the purpose of generating cDNA. Gene expression assays with FAM dye were purchased from Applied Biosystems (Foster City, CA., USA).

5. ELISA

Cytokine-specific enzyme-linked immunosorbent assays (ELISA) were used to quantify the amount of each cytokine (IL-6, IL-8, MCP-1 and M-CSF) secreted by NHDF cells after a period of being challenged with house dust mite extracts.

Cytokine-specific ELISA kits (DuoSet®ELISA Development System kits) were obtained from R&D Systems Inc. (Minneapolis, MN., USA).

Cytokine-specific ELISAs can be described as a sandwich ELISA, where a capture antibody (cytokine specific) binds to cytokines in the testing sample. This complex is then detected by a second cytokine detecting antibody. A substrate is then used to visualize the complex formed. Cytokine-specific ELISAs were carried out in flat bottomed 96 well microtiter plates. The plates were labeled based on the cytokine being tested. The following basic method of the various steps involved was as follows:

i. Coating

Coating antibody diluted according to supplier specifications was added to 11 ml of Phosphate Buffered Saline (PBS). The coating solution was then added to the corresponding microtiter plates (100 µl/well) and incubated overnight at 4°C. The plates were then washed three times the following day using a manual plate washer. The wash solution used was Phosphate Buffered Saline with Tween (PBST).

ii. Blocking

This step is essential in preventing non-specific binding of the antibody. A solution containing 1% Bovine Serum Albumin (BSA) added to Phosphate Buffered Saline (PBS) was used as the blocking solution. The plates were incubated for 1 hour at room temperature with 400 µl of blocking solution. Then the plate was washed with PBST three times.

iii. Loading the sample

A map of the 96 well plate was drawn to help with visualizing the location of the cytokine standard and the samples. A bullet tube rack for each 96 microtiter plate was then set up based on the map specifications. The cytokine standards (serially diluted) as well as the samples (both neat and diluted) were then added to the bullet tubes.

A multi-channel pipette was then used to transfer the standards and the samples for their corresponding bullet tubes to the cytokine specific plates. The samples were allowed to incubate at room temperature for 2 hours. The plates were then washed three times with PBST.

iv. Detection

A cytokine specific detecting antibody recognizes a different epitope from the cytokine specific capture antibody on the sample molecule. The cytokine detecting antibody also contains a biotin molecule attached to its heavy chain.

Cytokine specific antibody prediluted according to supplier specifications was added to 11ml of PBS. The solution was then added to the plate (100µl/well) and incubated for 2 hours at room temperature then the plate was washed three times with PBST.

v. Adding Horseradish Peroxidase- Streptavidin (SA-HRP)

Streptavidin (SA) binds to the biotin molecule bound detecting antibody. This increases the sensitivity of the cytokine-antibody complex.

SA-HRP was added to the plates (100µl/well) and allowed to incubate at room temperature for 20 minutes. The plate was then washed twice with PBST and twice with PBS.

vi. Substrate Addition

The substrate is essential to visualize SA-HRP bound cytokine-antibody complex. The substrate solution used was 3, 3', 5, 5'-Tetramethyle-benzadine (TMB). TMB was added row by row, paying attention to the time of addition. The reaction was allowed to occur until a color change (steel blue) was observed in the well with the highest concentration of standard.

vii. Reaction Stop

Once the steel-blue color change was observed, the reaction was stopped with sulfuric acid (H₂SO₄). The reaction was stopped one row at a time, making sure that each row had the same amount of reaction time. H₂SO₄ causes the reaction in each well to turn yellow.

The plates were read at a wavelength of 450 nm in a spectrophotometer.

6. NHDF cells' response to Lipopolysaccharide (LPS) Doses

Lipopolysaccharide (LPS) is a component of the outer membrane of gram negative bacteria. It is a known endotoxin that challenges cells through a mechanism involving Toll-Like Receptors (TLRs) (Cardoso et al., 2007). House dust mite (HDM) extracts do contain endotoxins that are likely to operate through toll-like receptors (Kauffman et al., 2006, Arlian et al., 2009). It is therefore essential to know how varying doses of LPS can affect NHDF cells. In a previous study by Arlian et al. (2008) a positive control of LPS (50 ng/ml) was used when NHDF cells were challenged with HDM extracts. However, it has not been determined if NHDF cells react differently when challenged by different doses of LPS.

The following method is used to challenge cells with different doses of LPS.

i. Growing NHDF Cells

NHDF cells were grown to confluence in T 75 flasks as described previously (Part B, point 1). Upon reaching confluence, the cells were harvested and used to seed 96 well microtiter plates to reach 60-70% confluence before being challenged with LPS doses.

ii. Making LPS Doses

A serial dilution ranging from 500,000 ng/ml to 0.12 ng/ml of sonicated LPS was carried out in cluster tubes purchased Corning Inc. (Corning, NY., USA).

The cluster tubes were placed in an arrangement similar to a 96 well microtiter plate. Consequently, there were 8 rows (A to H), each with 12 columns. Each dilution within this range was tested in quadruplicate. FGM-2 Media was used as a control and added to the first 4 tubes (A 1 to 4). The highest concentration of LPS was added to B 1 to 4 and the lowest concentration was H 9 to 12.

iii. Challenging Cells with LPS

The NHDF cells were challenged with LPS doses once the cells had reached 60-70% confluence.

The following method was used:

The medium in the 96 microtiter plate was aspirated and replaced with the serially diluted LPS in the cluster tubes.

The NHDF cells were challenged for 6 hours. This time point was selected as 6 hours were sufficient to induce cytokine secretion.

The supernatants from the wells were aspirated and stored in cluster tubes at -80°C in order to carry out cytokine- specific ELISAs for IL-6, IL-8, MCP-1 and M-CSF.

7. Testing the efficiency of using various concentrations of Polymyxin B (PmB) to bind LPS

Polymyxin B (PmB) is an antibiotic that is used to treat infections that are caused by gram negative bacteria. This antibiotic binds to negatively charged components of the Lipopolysaccharide (LPS) layer of the outer membrane and destabilizes it. PmB is also known to bind and inactivate endotoxins (Cardoso et al., 2007). Thus, when carrying out experiments where the activity of endotoxins needs to be inhibited, PmB is commonly used. A typical concentration of PmB used when working with house dust mite (HDM) extracts is 30µg/ml (Arlan et al., 2009). PmB was added to the extracts and incubated at 4°C for 18 hours before challenging cells.

Cells were challenged with varying concentrations of LPS + PmB in order to determine the best concentration of PmB to use in challenging cells that is sufficient at inactivating LPS without killing cells. *D. farinae* extract has 52, 115 EU/ 50 µg of extract, *D. pteronyssinus* extract has 8, 725 EU/ 50 µg of extract and *E. maynei* has 132, 569 EU/ 50 µg of extract. The LPS concentrations tested should encompass the different amounts of endotoxin found in the extracts. Thus, LPS concentrations of 1,500 EU/ml, 30,000

EU/ml, 120, 000 EU/ml were used in this assay. PmB concentrations used were doubling dilutions that ranged from 500,000 µg/ ml to 0 µg/ml.

NHDF cells were grown to confluence in T75 flasks (Part B, Point 1) and harvested cells were used to seed a 96 well microtiter plate. When the cells reached a 60-70% confluence, cells were challenged for 6 hours with LPS + PmB mixtures. The supernatants were collected in bullet tubes and used cytokine ELISAs.

NHDF cells were grown to confluence in T 75 flasks (Part B, Point 1) and harvested cells were used to seed a 96 well microtiter plate. When the cells reached a 60-70% confluence, cells were challenged for 6 hours with LPS + PmB mixtures. Cell confluence was established by visual observation of the distance between fibroblast cells. The supernatants were collected in bullet tubes and used cytokine ELISAs.

8. Challenging NHDF cells with HDM extracts that have been treated with PmB

House dust mite extracts contain endotoxins (Arlian et al., 2008). PmB is added to the HDM extracts with the intention that it will bind to endotoxin within the extract and inhibit endotoxin activity (Arlian et al. 2009). Thus, secreted cytokine (IL6, IL8, MCP-1 and M-CSF) after a period of being challenged with PmB treated extract will be due non endotoxin molecules in the extract.

NHDF cells were grown to confluence in T 75 flasks (Part B, Point 1) and harvested cells were used to seed a 96 well microtiter plate. When the cells reached a 60-70% confluence, cells were challenged for 6 hours with house dust mite extracts in the absence or presence of 50 µg/ml PmB. House dust mite extracts used to challenge the NHDF cells were added at 12.5 µg/ml, 25 µg/ml, 50 µg/ml or 100 µg/ml.

The supernatants were collected in bullet tubes and analyzed by cytokine ELISAs.

9. Modifying house dust mite extracts to remove the effect of proteins in the extract

House dust mite extracts contain molecules including proteins that are allergens. Some dust mite allergens exhibit protease activity like those that belong to group 1, 3, 6 and 9 (Thomas et al., 2010). Proteases act through protease activated receptors (PARs) to elicit immune responses (Thomas et al., 2010, Thomas et al., 2006, Kauffman et al., 2006 and Asokanthan et al., 2002). It is therefore essential to know how protein molecules in house dust mite extracts influence IL-6, IL-8, MCP-1 and M-CSF cytokine secretion by NHDF cells.

Two strategies were employed to study the role of protein molecules in house dust mite extracts. House dust mite extracts were boiled or treated with trypsin agarose.

i. Boiling house dust mite extracts

House dust mite extracts from *D. farinae*, *D. pteronyssinus* and *E. maynei* were boiled for 5 or 30 minutes. Five minutes of boiling is sufficient in denaturing proteins and 30 minutes of boiling would inactivate endotoxins in the extract (Gao et al., 2009).

The extracts were boiled using the following strategy:

One milliliter *D. farinae*, *D. pteronyssinus* and *E. maynei* extracts were boiled for 5 or 30 minutes in 2 ml eppendorf tubes.

The boiled extracts were spun for 10 minutes at 14.1 rcf to pellet the denatured proteins.

The supernatants were then pipetted into Pierce® Spin Columns (distributed by Thermo Scientific, MA, USA).

The supernatants were spun for 10 minutes at 14.1 rcf to ensure complete filtration.

The filtered extracts were stored at ~4°C.

Normal human dermal fibroblast (NHDF) cells were grown in 96 well microtiter plated to reach a 60- 70% confluence. NHDF cells were challenged for 6 hours with various

doses of extract $\pm 50 \mu\text{g/ml}$ PmB. Concentrations of extract used to challenge NHDF cells were $12.5 \mu\text{g/ml}$, $25 \mu\text{g/ml}$, $50 \mu\text{g/ml}$ or $100\mu\text{g/ml}$.

Following the 6 hour period of incubation, the supernatants were collected and used in cytokine ELISAs.

ii. Treating house dust mite extracts with trypsin agarose

Trypsin, a serine protease, digests polypeptides at the carboxyl ends of lysine or arginine amino acids. Digesting the house dust mite extracts into smaller peptide sequences is another method through which protein activity in inducing an immune response can be tested. Trypsin is known to disrupt adhesion between cells. Thus, by using trypsin agarose ensures that the trypsin can be removed from the extracts before being used to challenge the cells.

Extracts were digested with trypsin agarose using the following strategy:

$800 \mu\text{l}$ was added to a 1.5 ml eppendorf tube and washed with 1ml of qH_2O .

The resuspended trypsin agarose gel was then divided into three $500 \mu\text{l}$ aliquots. I.e. one for each house dust mite species.

Each gel aliquot was washed with 0.5 ml of qH_2O . This process was repeated 24 times.

The gel aliquots were washed with of 0.1 M NaHCO₃. This process was repeated twice.

The gel aliquots were combined and resuspended in 1 ml of NaHCO₃. The suspension was then dived into three 500 µl aliquots.

1 ml of each house dust mite extract was added to each tube.

The eppendorf tubes were taped to a rotating device and incubated at 37°C for 24 hours.

The incubated mixture was then centrifuged for 1 min at 14.1 rcf.

The supernatant was pipetted into Pierce[®] Spin Columns and spun for 1 minute at 1000g.

This step was repeated twice to ensure complete removal of trypsin agarose.

The filtered house dust mite extracts were stored at ~ 4°C.

Normal human dermal fibroblast (NHDF) cells were grown in 96 well microtitter plated to reach a 60- 70% confluence. NHDF cells were challenged for 6 hours with various doses of trypsin agarose treated extracts ± 50 µg/ml PmB. Concentrations of extract used to challenge NHDF cells were 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100µg/ml.

Following the 6 hour period of incubation, the supernatants were collected and used in cytokine ELISAs.

10. Looking at the protein profile for each mite extract

In order to determine if modifying the extract by boiling or treating with trypsin agarose affected the protein molecules found in the extract, a SDS- PAGE gel was run. A Western blot was also performed and probed with rabbit Ig antiserum to *D. farinae*, *D. pteronyssinus* and *E. maynei* house dust mites.

SDS-PAGE gel and Western blot were performed using standard laboratory protocols.

III. Results

A. Gene Expression

1. IL-6 Gene Expression by NHDF Cells in Response to Being Challenged with House Dust Mite Extract

Figure 1 illustrates IL-6 gene expression when adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml of *D. farinae* (DF), *D. pteronyssinus* (DP) or *E. maynei* (EM) extracts with or without LPS (50 ng/ml).

D. farinae extract induced IL-6 gene expression in a time dependent manner (Fig. 1). NHDF cells that were challenged for 24 hours expressed more IL-6 than cells challenged for 6 hours. When LPS was added to *D. farinae* extract, cells challenged for 24 hours showed the highest level of IL-6 gene expression than cells challenged for 6 or 12 hours. Boiled *D. farinae* extract did not induce IL-6 gene expression by NHDF cells over time. RQ values for cells challenged with boiled extract were comparable to constitutive amounts of expression at each time interval.

D. pteronyssinus extract it did not induce IL-6 gene expression by NHDF cells (Fig. 1). RQ values of cells challenged with *D. pteronyssinus* extract are comparable to constitutive amounts of gene expression at each time interval. When LPS was added to *D.*

pteronyssinus extract did not induce IL-6 secretion. IL-6 RQ values for cells challenged with boiled extract were comparable to constitutive RQ values.

E. maynei extract did not induce IL-6 gene expression by NHDF cells (Fig. 1). Gene expression values for cells challenged with extract are comparable to constitutive amounts of gene expression at each time interval. LPS when added to the extract did not enhance IL-6 gene expression. RQ values of cells challenged with extract + LPS were comparable to constitutive values of gene expression. Boiled extract did not seem to induce IL-6 gene expression. Cells challenged with boiled extract had IL-6 gene expression values comparable to constitutive amounts of expression.

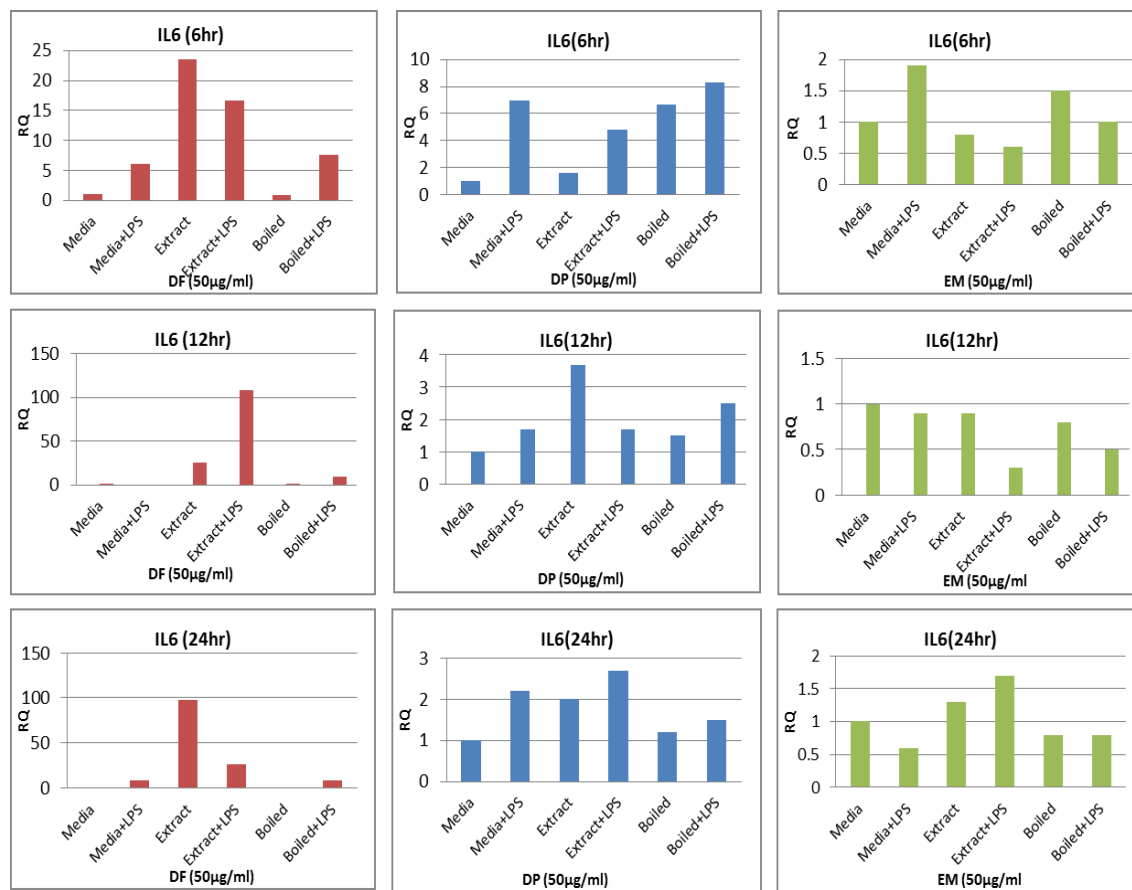


Figure 1: Relative quantification (RQ) values for IL6 gene expression after adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml house dust mite extract for 6, 12 or 24 hours. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Note, axes are different for each graph.

2. IL-8 Gene Expression by NHDF Cells in Response to Being Challenged with House Dust Mite Extract

Figure 2 illustrates IL-8 gene expression when adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml of *D. farinae* (DF), *D. pteronyssinus* (DP) or *E. maynei* (EM) extracts with or without LPS (50 ng/ml).

D. farinae extract induced IL-8 gene expression by NHDF cells (Fig. 2). IL-8 gene expression was higher than constitutive RQ values at each time interval. When LPS was added to *D. farinae* extract, IL-8 gene expression was enhanced at 12 and 24 hour time intervals. However, at 6 hours, when LPS was added to the extract, RQ values of IL-8 gene expression were lower than when extract alone was used to challenge the cells. Boiled extract did not induce IL-8 gene expression by NHDF cells. Thus, RQ values for IL-8 gene expression were comparable to constitutive amounts of expression at each time interval.

NHDF cells that were challenged with *D. pteronyssinus* extract for 12 hours induced IL-8 gene expression (Fig. 2). Gene expression values of cells challenged with extract are comparable to constitutive amounts of expression at 6 and 24 hour time intervals. When LPS was added to the extract, IL-8 gene expression was enhanced except at 12 hours. Boiling the extract did not reduce IL-8 gene expression. Gene expression values

for cells challenged with boiled extract for 6 and 24 time intervals were comparable to constitutive amounts of secretion at each time interval.

E. maynei did not induce IL-8 gene expression by NHDF cell (Fig. 2). RQ values for IL-8 gene expression was comparable to constitutive amounts of gene expression at each time interval. When LPS was added to the extract, IL-8 gene expression was not enhanced. RQ values for IL-8 gene expression when LPs was added to extract were comparable to constitutive amounts of expression. Boiled extract did not reduce IL-8 gene expression by NHDF cells. Gene expression values for boiled extract at each time interval were comparable to constitutive amounts of expression.

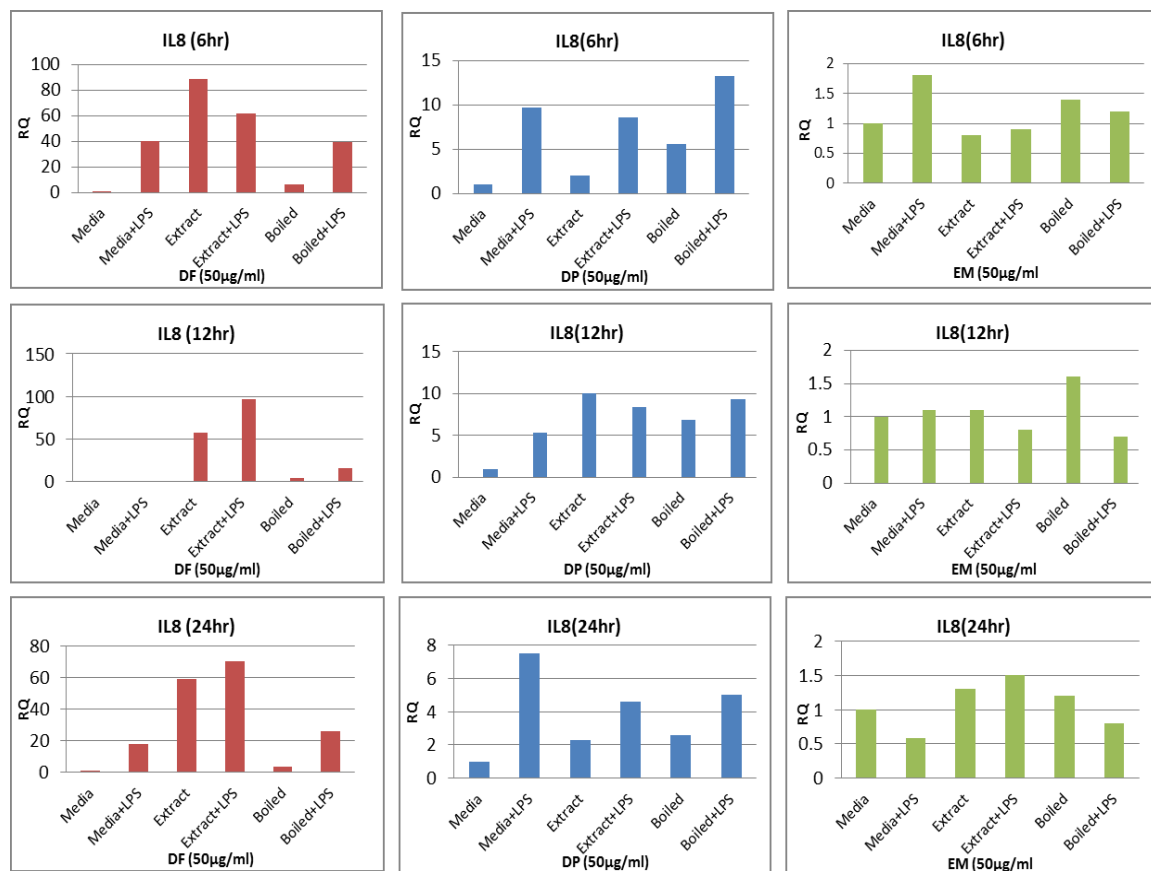


Figure 2: Relative quantification (RQ) values for IL8 gene expression after adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml house dust mite extract for 6, 12 or 24 hours. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Note, axes are different for each graph.

3. MCP-1 Gene Expression by NHDF Cells in Response to Being Challenged with House Dust Mite Extract

Figure 3 illustrates MCP-1 gene expression when adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml of *D. farinae* (DF), *D. pteronyssinus* (DP) or *E. maynei* (EM) extracts with or without LPS (50 ng/ml).

D. farinae extract induced MCP-1 gene expression (Fig. 3). At shorter time intervals MCP-1 gene expression is higher than at longer time intervals. When LPS was added to the extract, MCP-1 gene expression was affected. There was a down regulation of MCP-1 gene expression at 6 hours when LPS was added to *D. farinae* extract. However, when NHDF cells were challenged with DF extract + LPS for 12 or 24 hours the RQ values were comparable to cells challenged in the absence of LPS. Boiling the extract did not seem to stimulate gene expression. RQ values for MCP-1 gene expression by cells that were challenged with boiled extract were comparable to constitutive levels of expression at each time interval.

D. pteronyssinus extract did not induced MCP-1 gene expression by NHDF cells (Fig. 3). Gene expression values for cells that were challenged with *D. pteronyssinus* extract were comparable to constitutive amounts of expression. Boiling the extract did not reduce MCP-1 gene expression. RQ values of cells challenged with boiled extract in the

absence or presence of LPS were comparable to constitutive amounts of secretion at each time interval.

E. maynei extract did not induce MCP-1 gene expression by NHDF cells (Fig. 3). RQ values for MCP-1 gene expression when NHDF cells were challenged with *E. maynei* extract were comparable to constitutive amounts of expression at each time interval. LPS did not enhance MCP-1 gene expression at each time interval. RQ values for extract + LPS were comparable to constitutive amounts of secretion at each time interval. Challenging the cells with boiled extract did not influence MCP-1 gene expression.

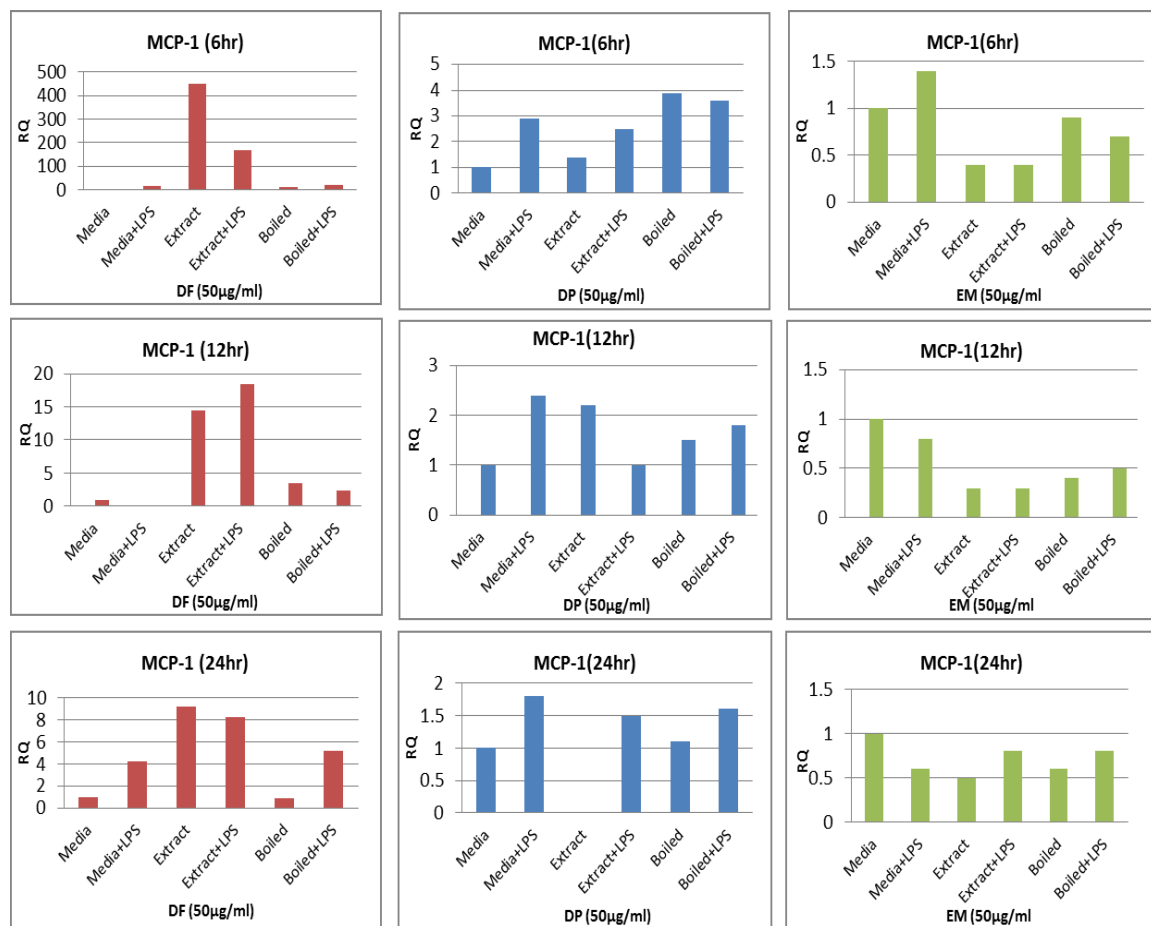


Figure 3: Relative quantification (RQ) values for MCP-1 gene expression after adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml house dust mite extract for 6, 12 or 24 hours. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Note, axes are different for each graph.

4. M-CSF Gene Expression by NHDF Cells in Response to Being Challenged with House Dust Mite Extract

Figure 4 illustrates M-CSF gene expression when adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml of *D. farinae* (DF), *D. pteronyssinus* (DP) or *E. maynei* (EM) extracts with or without LPS (50 ng/ml).

D. farinae extract induced some M-CSF gene expression at 6 hours but not at 12 or 24 hours (Fig. 4). Gene expression values for cells that were challenged with *D. farinae* extract were comparable to constitutive values of secretion at 12 and 24 hours. LPS when added to the extract did not enhance M-CSF gene expression at any time interval. Cells challenged with boiled extract reduced M-CSF gene expression compared to natural extract.

D. pteronyssinus extract did not induce M-CSF gene expression over time (Fig. 4). At each time interval, RQ values for M-CSF gene expression by cells that were challenged with *D. pteronyssinus* extract were comparable to constitutive amounts of expression. LPS did not enhance M-CSF gene expression. Cells that were challenged with DP + LPS had M-CSF gene expression values that were comparable to constitutive amounts of expression. Boiled extract does not seem to reduce or enhance M-CSF gene expression.

Cells that were challenged with boiled extract had RQ values that were comparable to constitutive amounts of expression at each time interval.

E. maynei extract did not induce or reduce M-CSF gene expression by NHDF cells over time (Fig. 4). Gene expression values for NHDF cells that were challenged with *E. maynei* extract were comparable to constitutive amounts of secretion at each time interval.

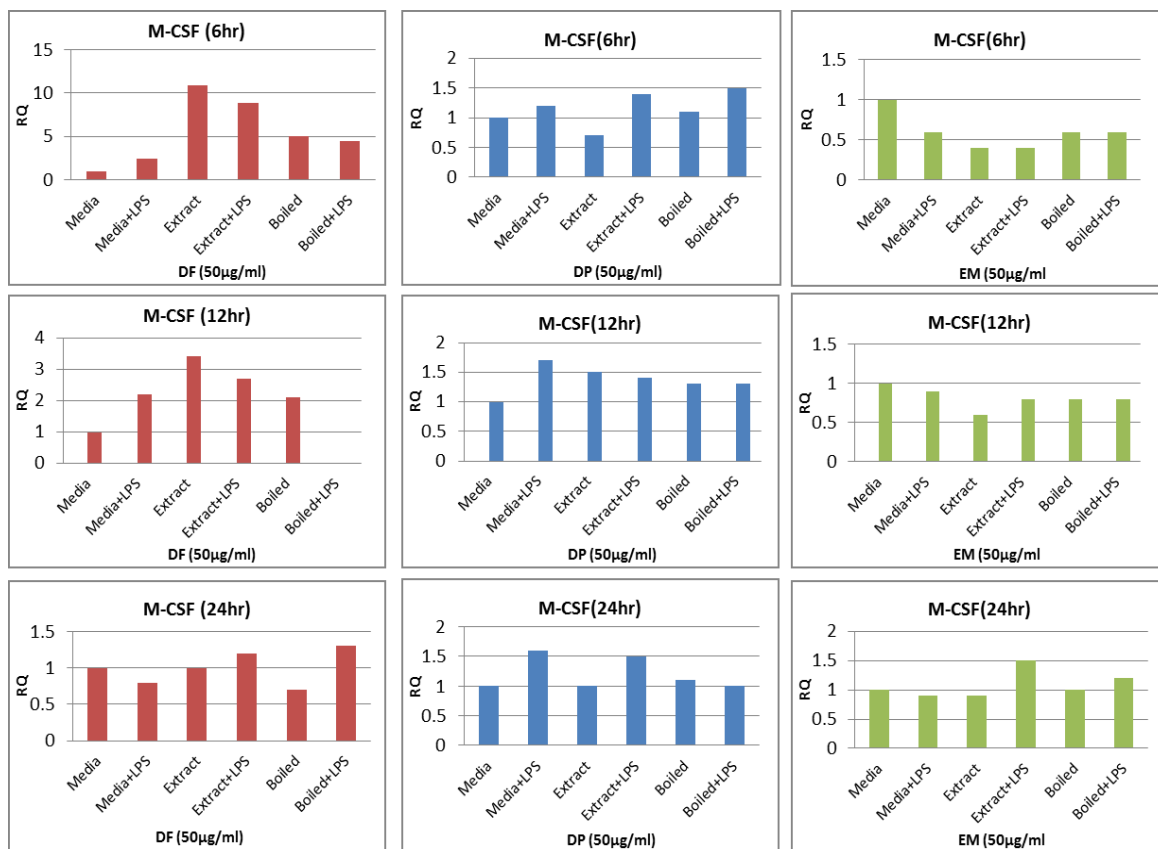


Figure 4: Relative quantification (RQ) values for M-CSF gene expression after adult normal human dermal fibroblast (NHDF) cells were challenged with 50 µg/ml house dust mite extract for 6, 12 or 24 hours. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Note, axes are different for each graph.

Summary

Gene Expression assays showed that *D. farinae* extract did induce cytokine gene expression. *D. pteronyssinus* and *E. maynei* extracts did not induce cytokine gene expression.

B. Normal Human Dermal Fibroblast (NHDF) Cell Response to Varying Doses of LPS

Normal Human Dermal Fibroblast (NHDF) cells were challenged with varying doses of LPS. This was examined in order to determine how cytokine secretion (IL-6, IL-8, MCP-1 and M-CSF) was affected by different concentrations of endotoxin. LPS (50 ng/ml) is also used as a control in experiments involving NHDF cells challenged with HDM extracts (Arlian et al. 2008). It is also essential to determine if this concentration was appropriate.

Figure 5 illustrates NHDF cells' response to being challenged for 6 hours with different concentrations of LPS (500,000 to 4.66×10^{-4} ng/ml). Constitutive secretion of IL-6, IL-8 and MCP-1 was negligible. IL-6, IL-8 and MCP-1 cytokine secretion by NHDF cells was detected using absorbance (450 nm) values. M-CSF cytokine secretion was also examined; however, absorbance values were negligible and not included in this figure.

Low and High doses of LPS resulted in increased secretion of IL-6, IL-8 and MCP-1 by NHDF cells. Peak secretions of IL-6, IL-8 and MCP-1 were induced by NHDF cells that were challenged with 2.98×10^{-2} ng/ml and 5.0×10^5 ng/ml LPS. The lowest amounts of IL-6 and IL-8 were secreted by cells that were challenged with 61.04 ng/ml LPS. The lowest amount of MCP-1 was secreted by cells that were challenged with 122 ng/ml LPS.

An appropriate dose of LPS to stimulate NHDF cells was determined based on IL-6, IL-8 and MCP-1 cytokine absorbance values. It was determined that 50 ng/ml LPS would be an appropriate dose to challenge NHDF cells. It was established that this dose would be low enough to induce cytokine secretion without overshadowing any cytokine secretion that is a result of being challenged with house dust mite extracts.

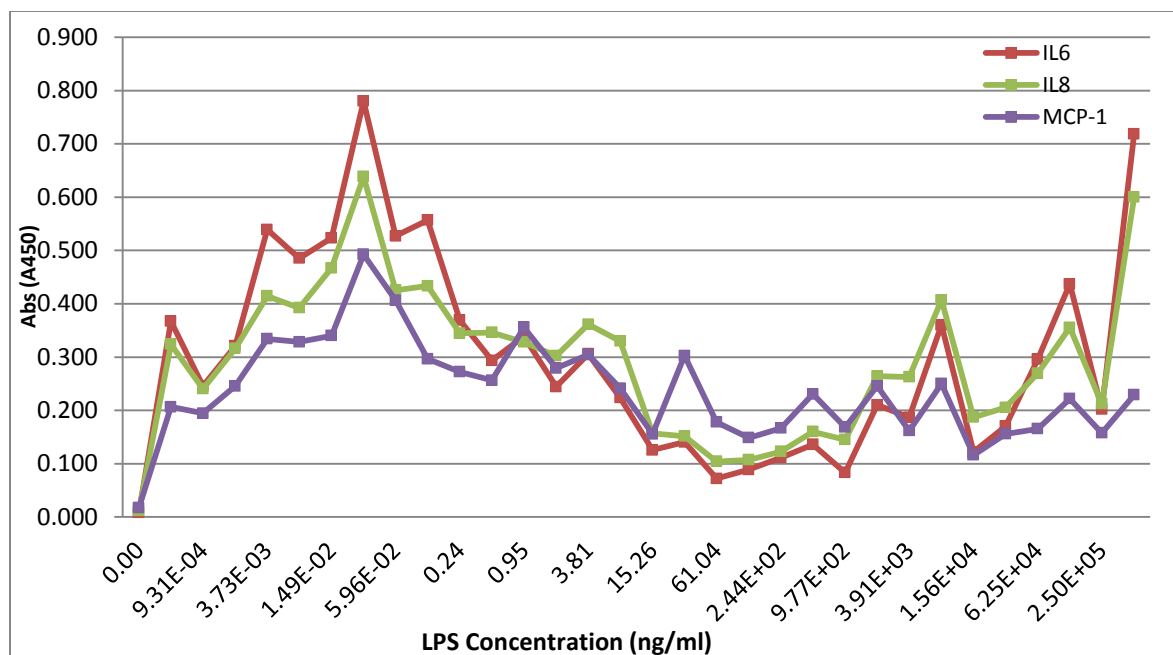


Figure 5 Normal Human Dermal Fibroblast (NHDF) cells were challenged for 6 hours with varying doses of LPS (ng/ml). Data are average absorbance values (n=3) from one of two experiments.

C. NHDF Cells Response to Low Doses of House Dust Mite Extract

1. IL-6 Secretion by NHDF Cells in Response to Varying Doses of House Dust Mite Extract

Figure 6 illustrates mean \pm SD values for IL-6 cytokine secretion by normal dermal fibroblast cells (NHDF) after being challenged with house dust mite extracts in the absence or presence of LPS (50 ng/ml) at 6, 12 and 24 hour time intervals. The house dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). The concentrations of house dust mite extracts used to challenge the cells were 0, 5, 10 and 20 μ g/ml. NHDF cells were also challenged for 1 hour; however mean values of secretion at this time interval were negligible and thus not included in this figure.

D. farinae extract induced IL-6 secretion by NHDF cells in a dose and time dependent manner (Fig. 6). Higher amounts of IL-6 were secreted by NHDF cells that were challenged with higher doses of extract and for longer times. LPS when added to the varying doses of *D. farinae* extract enhanced IL-6 secretion above that induced by DF extract alone. Thus, 20 μ g/ml DF + LPS showed higher levels of secretion when compared to 0, 5, 10 μ g/ml of DF + LPS.

D. pteronyssinus extract did not seem to induce IL-6 secretion by NHDF cells (Fig. 6). Cells that were challenged with 5 µg/ml DP extract for 6 hours showed some IL-6 secretion, however, cells challenged for 12 and 24 hours with the same concentration of extract did not secrete any IL-6. When LPS was added to the various doses of DP extract, IL-6 secretion was induced.

E. maynei extract induced IL-6 secretion by NHDF cells in a dose and time dependent manner (Fig. 6). NHDF cells that were challenged with higher doses of extract and for longer time intervals secreted more IL-6 than cells that were challenged with lower doses for shorter time intervals. When LPS was added to the various doses of EM extract, IL-6 secretion was enhanced above that induced by EM extract alone.

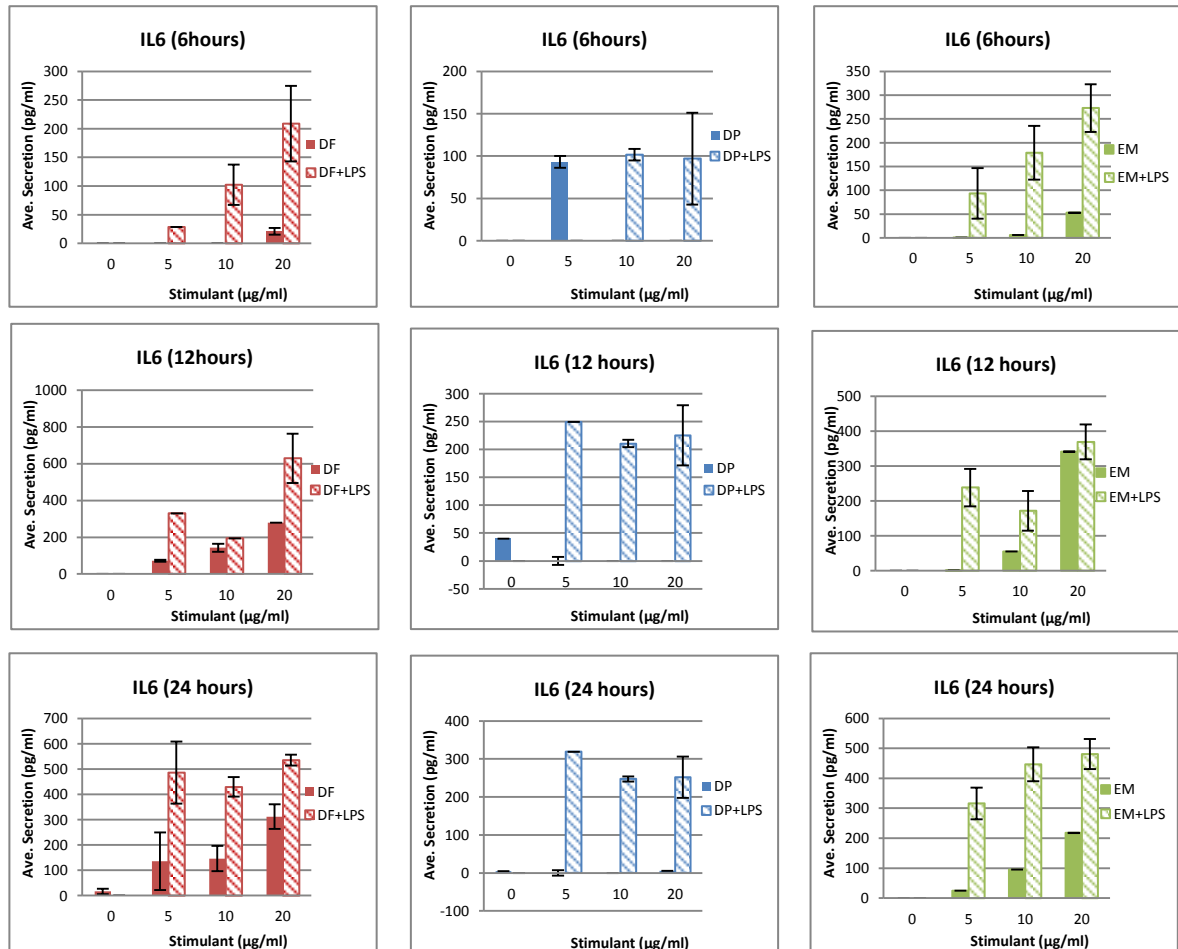


Figure 6 IL-6 secretion by normal dermal fibroblast (NHDF) cells after being challenged for 6, 12 and 24 hours with varying doses of house dust mite extract with and without LPS (50 ng/ml). House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Data are means \pm SD (n=2). Note, axes are different for each graph.

2. IL-8 Secretion by NHDF Cells in Response to Varying Doses of House Dust Mite Extract

Figure 7 illustrates mean \pm SD values for IL-8 cytokine secretion by normal dermal fibroblast cells (NHDF) after being challenged with house dust mite extracts in the absence or presence of LPS (50 ng/ml) at 6, 12 and 24 hour time intervals. The house dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). The concentrations of house dust mite extracts used to challenge the cells were 0, 5, 10 and 20 μ g/ml. NHDF cells were also challenged for 1 hour; however mean values of secretion at this time interval were negligible and thus not included in this figure.

D. farinae extract induced IL-8 secretion by NHDF cells in a dose and time dependent manner (Fig. 7). A higher amount of IL-8 was secreted by NHDF cells that were challenged with higher doses of extract for longer time intervals. LPS when added to the varying doses of *D. farinae* extract enhanced IL-8 secretion above that induced by DF extract alone for 6 hours. When cells were challenged for 12 or 24 hours with DF + LPS, IL-8 secretion was comparable to amounts secreted in the absence of LPS.

D. pteronyssinus extract did not seem to induce IL-8 secretion by NHDF cells (Fig. 7). Cells that were challenged with 5 µg/ml DP extract for 6 hours showed some IL-8 secretion, however, cells challenged for 12 and 24 hours with the same concentration of extract did not secrete any IL-8. When LPS was added to the various doses of DP extract, IL-8 secretion was induced.

E. maynei extract induced IL-8 secretion by NHDF cells in a dose and time dependent manner (Fig. 7). The highest amount of IL-8 was secreted when NHDF cells were challenged with 20 µg/ml of extract for 12 hours. When LPS was added to the various doses of EM extract, IL-8 secretion was enhanced except when the cells were challenged for 24 hours with 20µg/ml of EM + LPS.

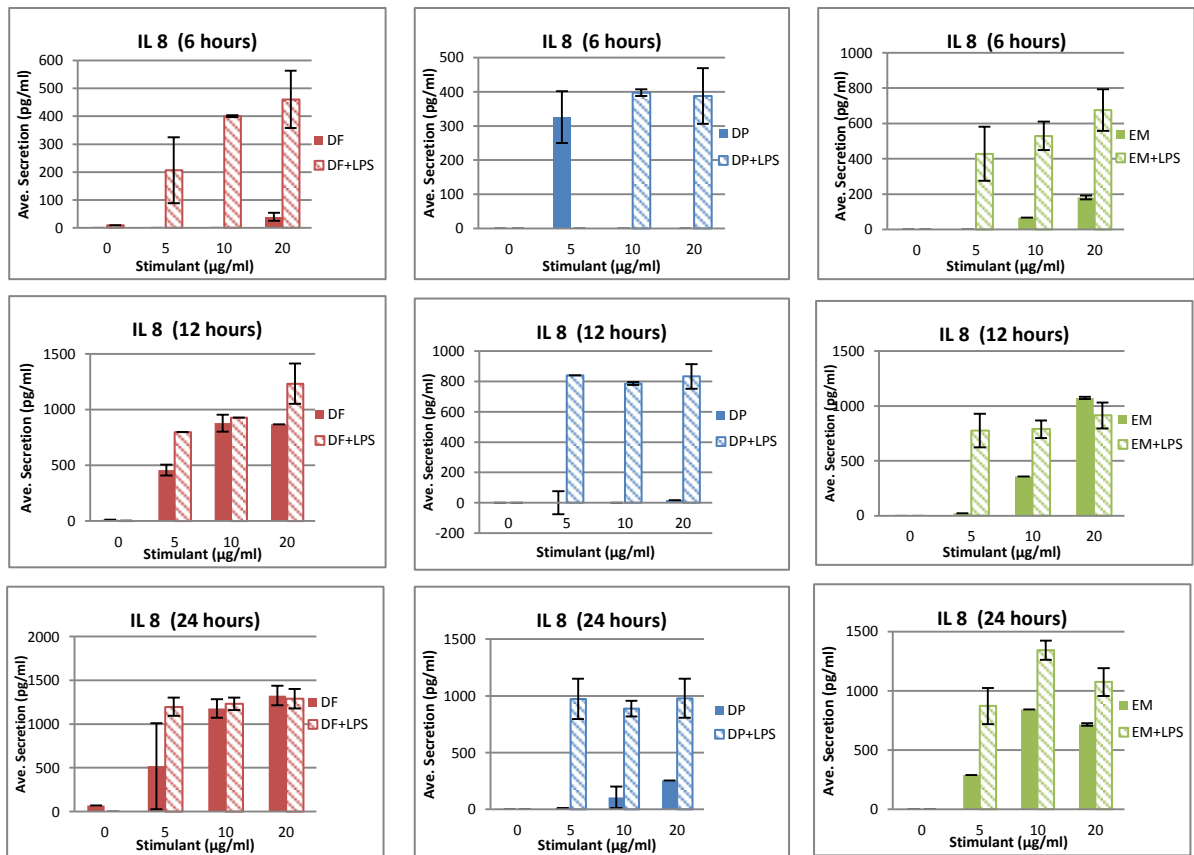


Figure 7 IL-8 secretion by normal dermal fibroblast (NHDF) cells after being challenged for 6, 12 and 24 hours with varying doses of house dust mite extract with and without LPS (50 ng/ml). House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Data are means \pm SD (n=2). Note, axes are different for each graph.

3. MCP-1 Secretion by NHDF Cells in Response to Varying Doses of House Dust Mite Extract

Figure 8 illustrates mean \pm SD values for MCP-1 cytokine secretion by normal dermal fibroblast cells (NHDF) after being challenged with house dust mite extracts in the absence or presence of LPS (50 ng/ml) at 6, 12 and 24 hour time intervals. The house dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). The concentrations of house dust mite extracts used to challenge the cells were 0, 5, 10 and 20 μ g/ml. NHDF cells were also challenged for 1 hour; however mean values of secretion at this time interval were negligible and thus not included in this figure.

D. farinae extract induced MCP-1 secretion by NHDF cells in a dose dependent manner (Fig. 8). LPS (50 ng/ml) when added to the varying doses of *D. farinae* extract did not enhance MCP-1 secretion above that induced by DF extract alone.

D. pteronyssinus extract induced MCP-1 secretion by NHDF cells in a time dependent manner (Fig. 8). Cells that were challenged for 24 hours showed more MCP-1 secretion than cells challenged with varying doses for shorter time intervals. When LPS (50 ng/ml) was added to the various doses of DP extract, MCP-1 secretion was higher than cells that were challenged with DP alone.

E. maynei extract induced MCP-1 secretion by NHDF cells in a dose and time dependent manner (Fig. 8). Cells challenged with 10 µg/ml and 20 µg/ml of extract for 24 hours secreted similar amounts of MCP-1. When LPS was added to the extract, MCP-1 secretion increased except when cells were challenged for 12 or 24 hours with 20 µg/ml EM+LPS.

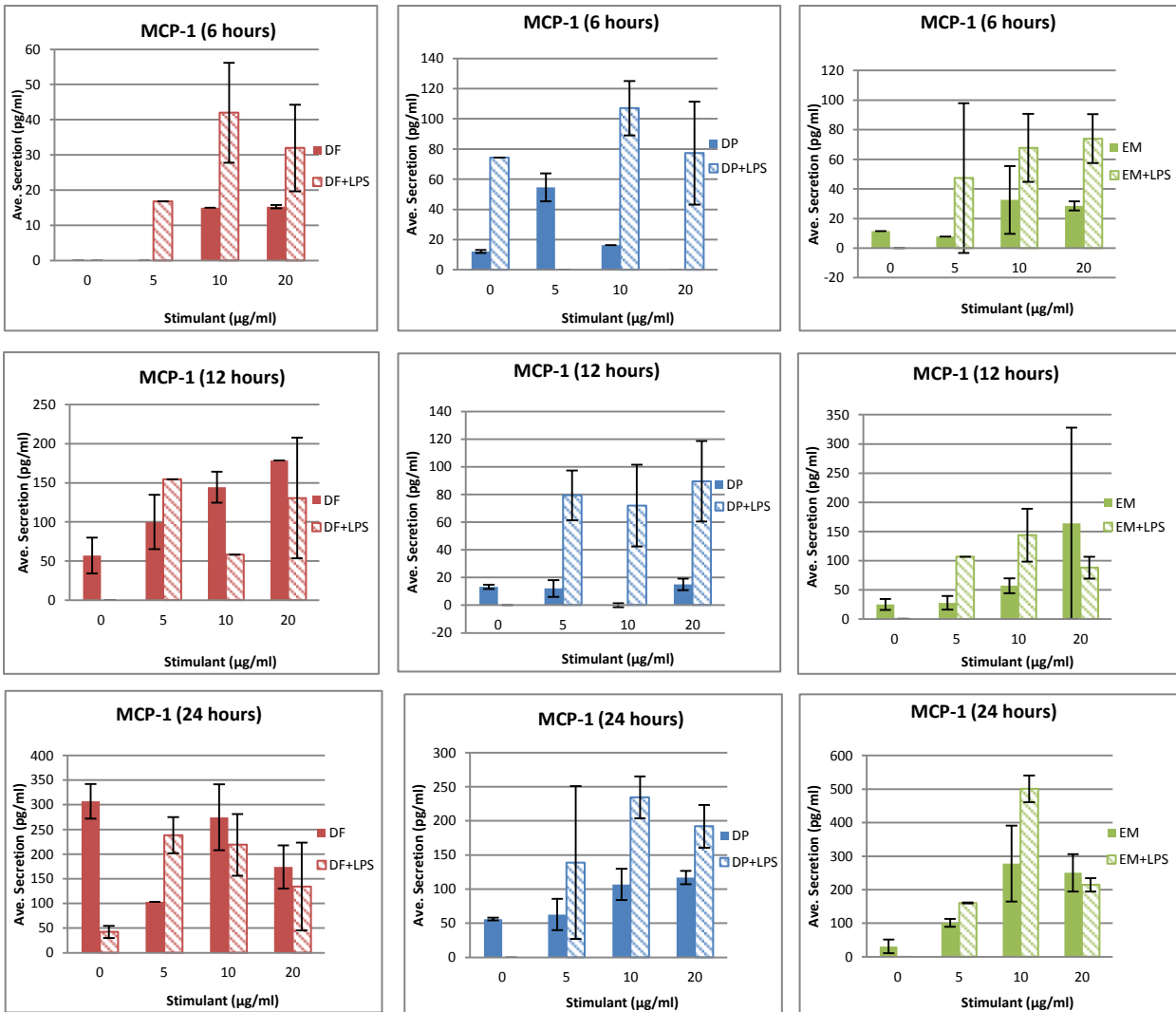


Figure 8 MCP-1 secretion by normal dermal fibroblast (NHDF) cells after being challenged for 6, 12 and 24 hours with varying doses of house dust mite extract with and without LPS (50 ng/ml). House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Data are means \pm SD (n=2). Note, axes are different for each graph.

Summary

In summary, low doses of each house dust mite extract influenced cytokine secretion by NHDF cells differently. *D. farinae* and *E. maynei* extracts modulated cytokine secretion in a dose and time dependent manner. The results also show that *D. pteronyssinus* extract did not modulate IL-6 and IL-8 cytokine secretion.

D. Modulatory Effect of Endotoxins in Extracts

1. Normal Human Dermal Fibroblast (NHDF) cell Response to PmB Treated Endotoxin

PmB is an antibiotic that is known to inactivate endotoxins (Cardoso et al., 2007). In order to determine the appropriate concentration that would inactivate endotoxins found in house dust mite extract, various concentrations of PmB (500 µg/ml to 0.98 µg/ml) were used to treat three different LPS concentrations. IL-6, IL-8 and MCP-1 cytokine secretion was detected using absorbance (450 nm) values. M-CSF cytokine secretion was also detected; however, the values were negligible and not included in the figure.

Figure 9, demonstrates the response of Normal Human Dermal Fibroblast (NHDF) cells to three doses of endotoxin treated with varying doses of polymyxin B (PmB) for 6 hours. The three LPS concentrations used were 1,500 EU/ml (50 ng/ml), 30,000 EU/ml

(1,000 ng/ml) and 120, 000 EU/ml (4,000 ng/ml). LPS alone stimulated secretion of IL-6, IL-8 and MCP-1 by NHDF cells. NHDF cells that were challenged with LPS and PmB showed decreased secretion of IL-6, IL-8 and MCP-1 compared to cells that were challenged with LPS alone (Fig 9). A PmB dose of 0.98 µg/ml eliminated almost all the stimulating effect of LPS.

Based on these results, a PmB dose of 50 µg/ml was determined as an appropriate dose to inactivate endotoxins found in house dust mite extracts without causing cells to die.

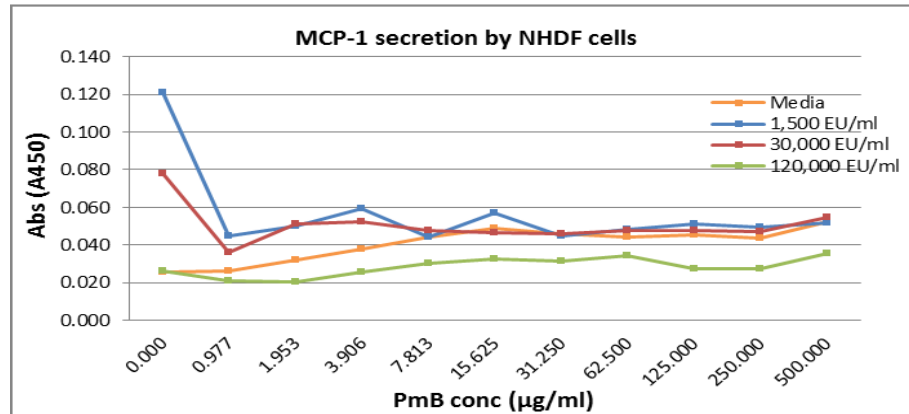
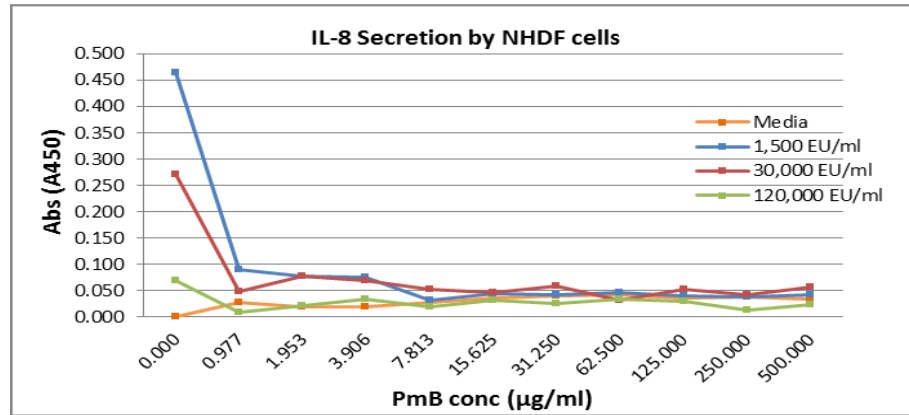
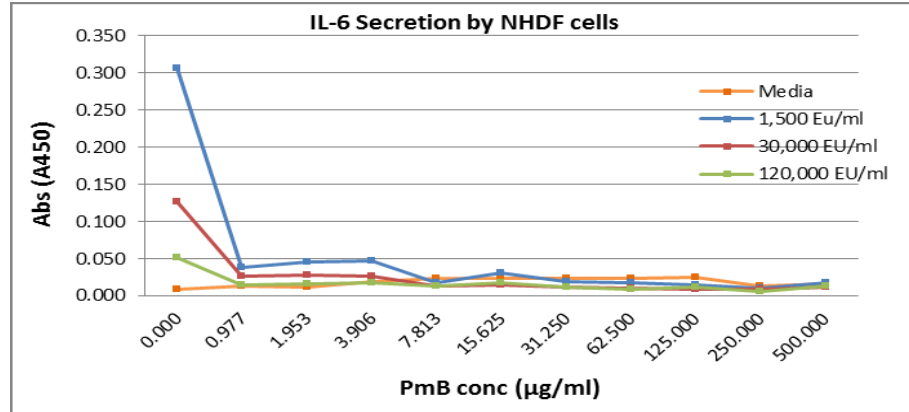


Figure 9 Normal Human Dermal Fibroblast (NHDF) cells were challenged for 6 hours with varying doses of endotoxin in the absence or presence of PmB. Data are means (n=2).

2. The Modulatory Effect of Varying Doses of PmB Treated House Dust Mite extract on Cytokine Secretion

Normal human dermal fibroblast (NHDF) cells were challenged with varying doses of house dust mite extract in the absence or presence of polymyxin B (PmB) (Fig 10). House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Extract concentrations used were 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. PmB concentration used was 50 µg/ml. For controls, NHDF cells were also challenged for 6 hours with three different concentrations of LPS in the absence or presence of PmB.

In 50 µg/ml of DF extract there was 52,115 EU/ml. In 50 µg/ml of DP extract there was 8,725 EU/ml. In 50 µg of EM extract there was 132,569 EU/ml. LPS concentrations used to challenge the cells were 50 ng/ml (1,500 EU/ml), 1,000 ng/ml (30,000 EU/ml) and 4,000 ng/ml (120,000 EU/ml). For all the experiments, NHDF cells were challenged for 6 hours and cytokine secretion for IL-6, IL-8 and MCP-1 was determined. M-CSF cytokine secretion was negligible and therefore, data were not included.

D. farinae, *D. pteronyssinus* and *E. maynei* extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig 10). The amount of IL-6 secreted for cells challenged with house dust mite extract was higher than for NHDF cells that were challenged with

varying doses of LPS. NHDF cells that were challenged with PmB treated extracts secreted less IL-6 than cells treated with untreated extract.

D. farinae and *D. pteronyssinus* extracts induced IL-8 secretion by NHDF cells in a dose dependent manner (Fig 10). The amount of IL-8 secretion was greater for cells challenged with house dust mite extracts compared to cells challenged with varying doses of LPS. NHDF cells that were challenged with PmB treated *D. farinae* extract secreted a low amount of IL-8 compared to cells challenged with PmB treated extracts.

NHDF cells secretion of IL-8 was affected by varying doses of *E. maynei* (Fig. 10). NHDF cells that were challenged with PmB treated *E. maynei* extract secreted lower amounts of IL-8 to those cells that were treated with extract alone.

D. farinae extract stimulated secretion of MCP-1 secretion from NHDF cells in a dose dependent manner (Fig. 10). The amount of MCP-1 secreted when challenged with house dust mite extract was higher than for NHDF cells challenged with varying doses of LPS alone. Treating DF extract with PmB resulted in a loss of the ability of the extract to induce secretion of MCP-1.

Varying doses of *D. pteronyssinus* and *E. maynei* extracts did not induce MCP-1 secretion by NHDF cells in a dose dependent manner (Fig. 10). NHDF cells that were challenged

with PmB treated *D. pteronyssinus* and *E. maynei* extracts secreted similar amounts of MCP-1 to those cells that were treated with extract alone.

Summary

In summary, *D. farinae* and *D. pteronyssinus* extracts treated with PmB did not induce IL-6 or IL-8 secretion. However, *D. farinae* and *D. pteronyssinus* extracts treated with PmB did induce MCP-1 secretion. *E. maynei* extract treated with PmB did not influence IL-6, IL-8 or MCP-1 cytokine secretion which is indicative that other molecules that are not endotoxins in the extract are involved in cytokine secretion.

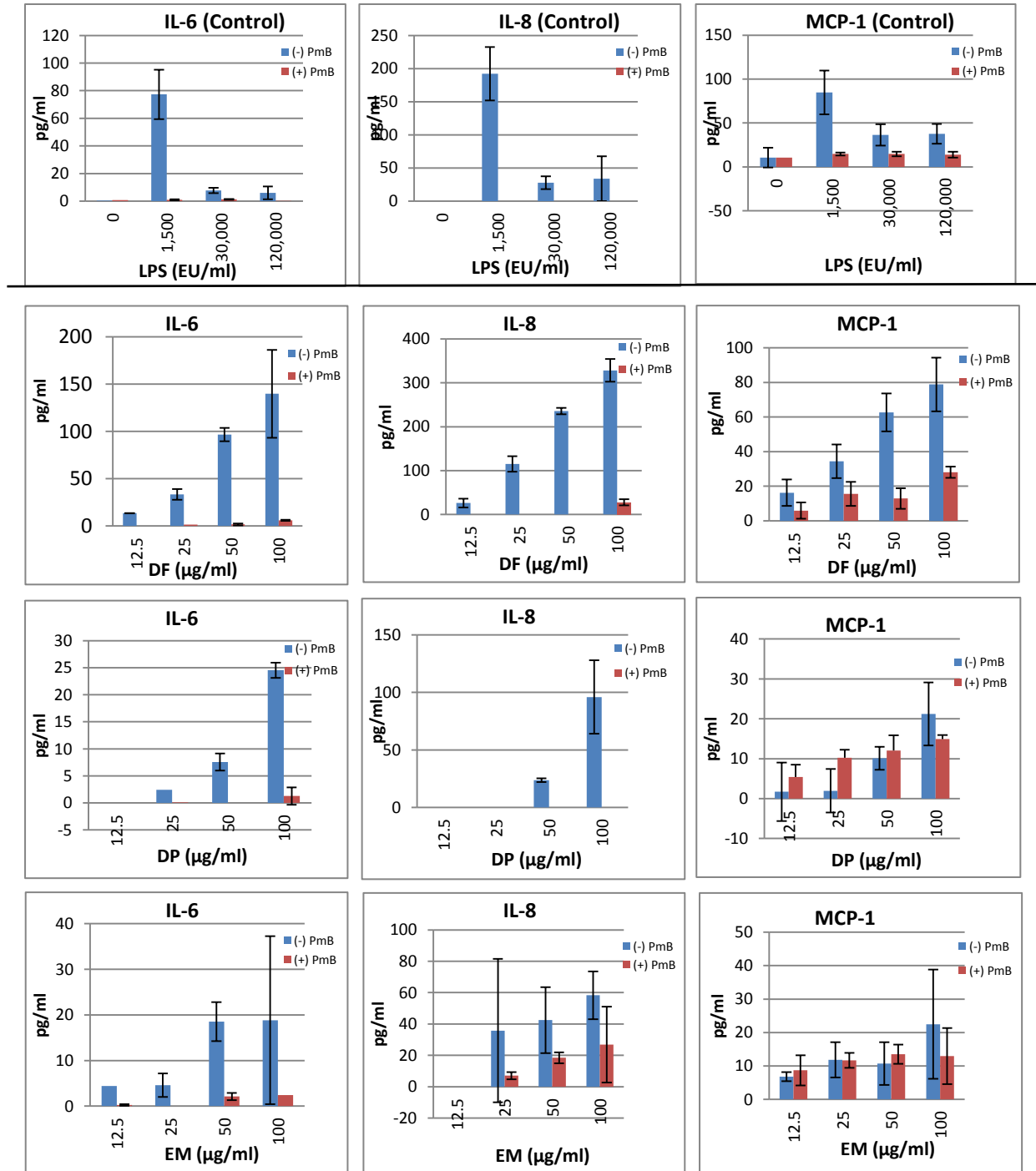


Figure 10 : Modulatory effect of varying doses of house dust mite extract on IL-6, IL-8 and MCP-1 secretion by normal human dermal fibroblast cells (NHDF) in the absence or presence of PmB. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Data are means \pm SD (n=3). Note, axes are different for each graph.

E. Modulatory Effect of Proteins in Extracts

1. Looking at the allergen profile of HDM extracts used to challenge NHDF cells

D. farinae (DF), *D. pteronyssinus* (DP) and *E. maynei* (EM) extracts, boiled extracts (5, 30 mins) and extracts digested with trypsin agarose were run on a SDS-PAGE gel (Fig. 11a). The protein profiles of the extracts were different from the same extracts that were boiled for 5, 30 minutes or digested with trypsin agarose. Extracts boiled for 5 or 30 minutes resulted in heat labile proteins in the extract being denatured. Trypsin, a serine protease, digested the proteins in the extract thus resulting in a protein profile different from extract that was not digested.

A Western blot analysis using rabbit immunoglobulin g (IgG) serum was used to probe against DF, DP and EM (Fig. 11b). The Western blot shows that IgG produced in response to DF, DP and EM was detected in the extracts as well as the modified extracts.

- a**
- Lane 1: Std
- Lane 2: DF
- Lane 3: 5min Boiled
- Lane 4: 30min Boiled
- Lane 5: Try-Ag
- Lane 6: DP
- Lane 7: 5 min Boiled
- Lane 8: 30 min Boiled
- Lane 9: Try-Ag
- Lane 10: EM
- Lane 11: 5 min Boiled
- Lane 12: Try-Ag

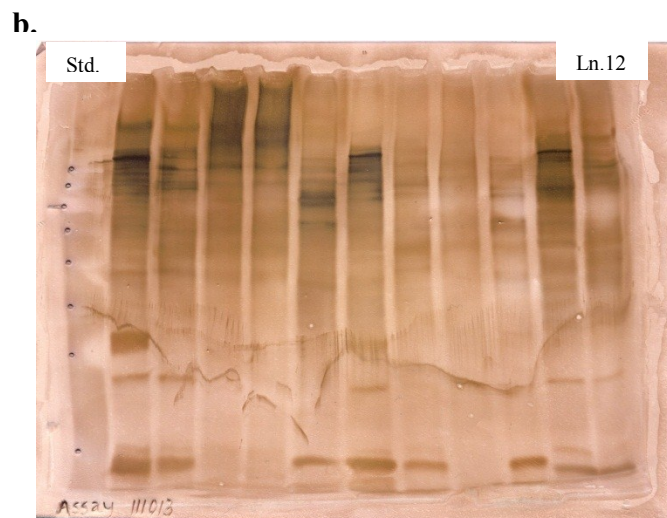
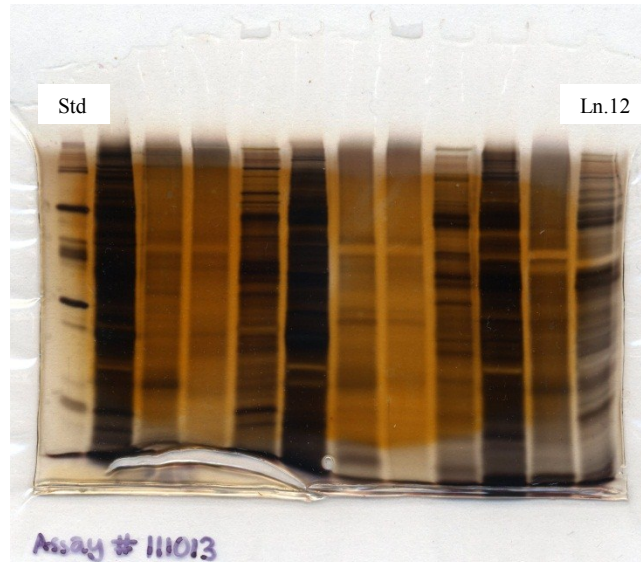


Figure 11 Protein profile of house dust mite extracts. SDS-PAGE gel (a.) of house dust mite extracts untreated, boiled or digested with trypsin agarose. Western blot (b.) using rabbit antiserum against DF, DP and EM. DF= *D. farinae*, DP= *D. pteronyssinus* and EM= *E. maynei*.

2. The Modulatory Effect of Varying Doses of Boiled House Dust Mite extract on Cytokine Secretion

Normal human dermal fibroblast (NHDF) cells were challenged with varying doses of boiled and unboiled house dust mite extract in the absence or presence of polymyxin B (PmB) (Fig. 12). House dust mite extracts were boiled for 5 mins or 30 mins. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Extract concentrations used were 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. NHDF cells were challenged for 6 hours and cytokine secretion for IL-6 and IL-8 were determined. MCP-1 and M-CSF cytokine secretion was negligible and, therefore, not included.

D. farinae treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extract that was boiled for 5 minutes, the amount of IL-6 secreted was reduced. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted very low amounts of IL-6. IL-6 secretion was undetectable by cells that were challenged with boiled and unboiled extract in the presence of PmB.

D. pteronyssinus treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extract that was boiled

for 5 minutes, the amount of IL-6 secreted was comparable to unboiled extract. The amount of IL-6 secreted by cells challenged with 100 µg/ml of extract boiled for 5 minutes secreted more IL-6 than unboiled extract of the same concentration. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted very low amounts of IL-6. IL-6 secretion was undetectable by cells that were challenged with boiled and unboiled extract in the presence of PmB.

E. maynei treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extract that was boiled for 5 minutes, the amount of IL-6 secreted was higher than the amount secreted after exposure to unboiled extract. The amount of IL-6 secreted by cells challenged with 100 µg/ml of extract boiled for 5 minutes secreted more IL-6 than unboiled extract of the same concentration. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted very low amounts of IL-6. IL-6 secretion was undetectable by cells that were challenged with boiled and unboiled extract in the presence of PmB.

D. farinae and *E. maynei* extracts affected IL-8 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extracts that were boiled for 5 minutes, the amount of IL-8 secreted was comparable to unboiled extract. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted very low

amounts of IL-8. PmB treated *D. farinae* extract had no effect on IL-8 secretion at low concentrations (12.5 µg/ml and 25 µg/ml).

D. pteronyssinus treated extract affected IL-8 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extract that was boiled for 5 minutes, the amount of IL-8 secreted was comparable to unboiled extract. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted very low amounts of IL-8. When PmB was added to the extracts, IL-8 secretion was undetectable.

E. maynei treated extract affected IL-8 secretion by NHDF cells in a dose dependent manner (Fig. 12). When cells were challenged with extract that was boiled for 5 minutes, the amount of IL-8 secreted was higher than the amount secreted by unboiled extract. NHDF cells that were challenged with extract that was boiled for 30 minutes secreted comparable amounts of IL-8 to those cells challenged with unboiled extract. Cells that were challenged with PmB treated unboiled and boiled extract, did not noticeably reduce IL-8 secretion.

Summary

In summary, house dust mite extracts boiled for 5 minutes still induced IL-6 and IL-8 secretion. Therefore, heat labile proteins were not essential for inducing cytokine

secretion. Extracts boiled for 30 minutes exhibited decreased IL-6 and IL-8 secretion. However, neutralized endotoxins and proteins did not remove stimulatory properties of *D. pteronyssinus* and *E. maynei* extracts.

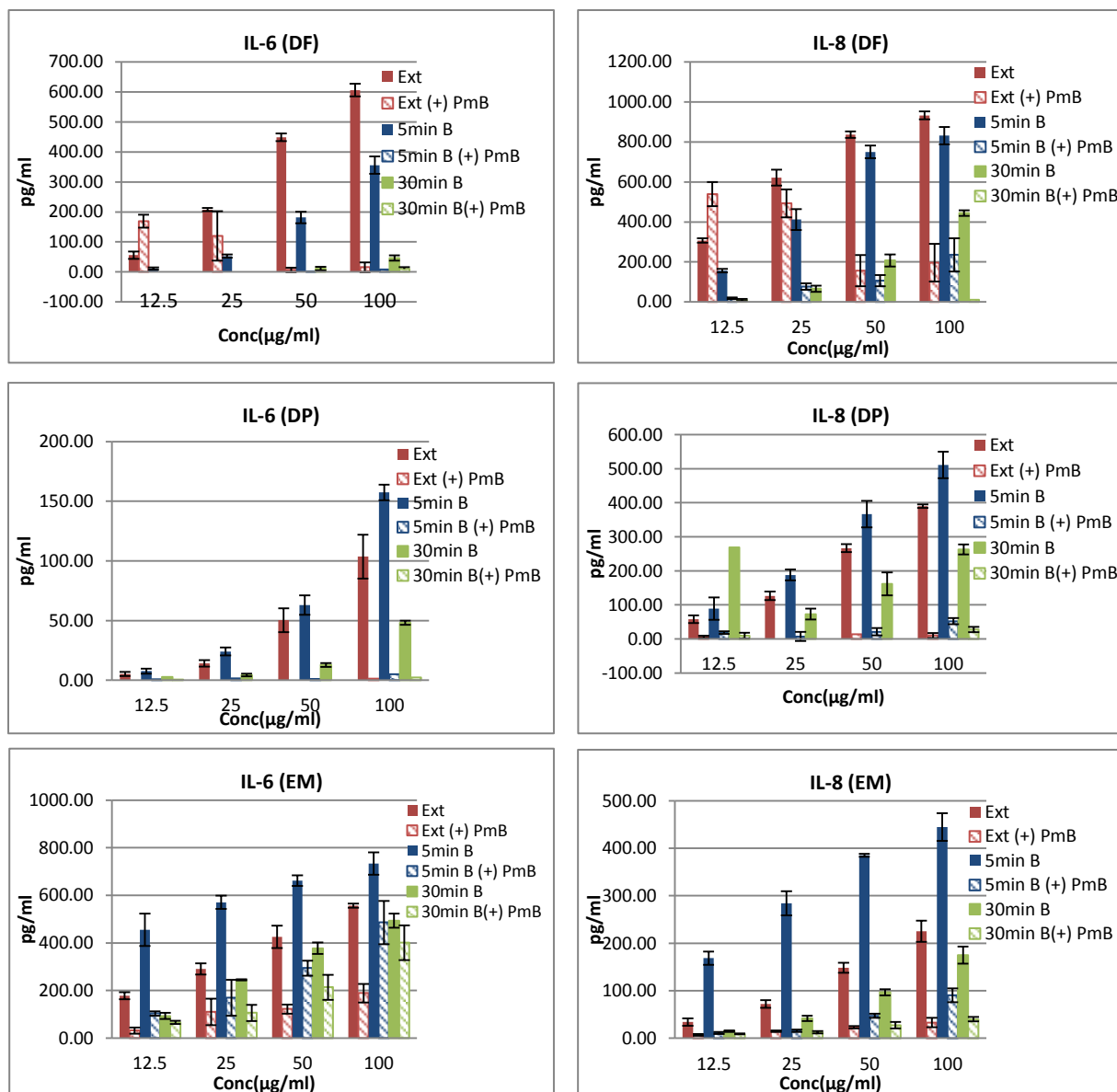


Figure 12 Modulatory Effect of varying doses of boiled and unboiled extract of the secretion of IL-6 and IL-8 by normal human dermal fibroblast (NHDF) cells. DF= *D. farinae*, DP= *D. pteronyssinus*, EM= *E. maynei*. Data are means \pm SD (n=3). Note, axes are different for each graph.

3. The Modulatory Effect of Varying Doses of Trypsin- Agarose treated House Dust Mite extract on Cytokine Secretion

Normal human dermal fibroblast (NHDF) cells were challenged with varying doses of trypsin- agarose treated extract as well as untreated extract in the absence or presence of polymyxin B (PmB) (Fig. 13). House dust mite extracts were treated with trypsin- agarose overnight. House dust mite extracts used were *Dermatophagoides farinae* (DF), *Dermatophagoides pteronyssinus* (DP) and *Euroglyphus maynei* (EM). Extract concentrations used were 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. NHDF cells were challenged for 6 hours and cytokine secretion for IL-6 and IL-8 was determined. MCP-1 and M-CSF cytokine secretion was negligible and therefore, not included.

D. farinae treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 13). When cells were challenged with trypsin agarose, the amount of IL-6 secreted was reduced. When PmB was added to the extracts, IL-6 secretion was undetectable.

D. pteronyssinus treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 13). When cells were challenged with trypsin agarose, the amount of IL-6 secreted was reduced. IL-6 secretion was undetectable by cells that were

challenged by untreated extract and trypsin agarose treated extract in the presence of PmB.

E. maynei treated extract affected IL-6 secretion by NHDF cells in a dose dependent manner (Fig. 13). When cells were challenged with trypsin agarose, the amount of IL-6 secreted was comparable amounts secreted by cells challenged with untreated extract. In the presence of PmB IL-6 secretion was reduced by cells that were challenged by untreated and trypsin agarose treated extract. IL-6 secretion was not affected by NHDF cells that were challenged with extract + PmB.

D. farinae and *E. maynei* treated extract affected IL-8 secretion by NHDF cells in a dose dependent manner (Fig. 13). When cells were challenged with trypsin agarose, the amount of IL-8 secreted was comparable to untreated extract. IL-8 secretion was reduced by cells that were challenged by untreated extract and trypsin agarose treated extract in the presence of PmB.

D. pteronyssinus treated extract affected IL-8 secretion by NHDF cells in a dose dependent manner (Fig. 13). When cells were challenged with trypsin agarose, the amount of IL-8 secreted was reduced. IL-8 secretion was very low by cells that were

challenged by untreated extract and trypsin agarose treated extract in the presence of PmB.

Summary

In summary, *D. farinae* and *D. pteronyssinus* trypsin agarose treated extracts caused a decrease in IL-6 and IL-8 cytokine secretion. *E. maynei* trypsin agarose treated extracts did not affect IL-6 and IL-8 cytokine secretion. This result implied that other aqueous molecules in the extract that work through other cellular pathways may be involved.

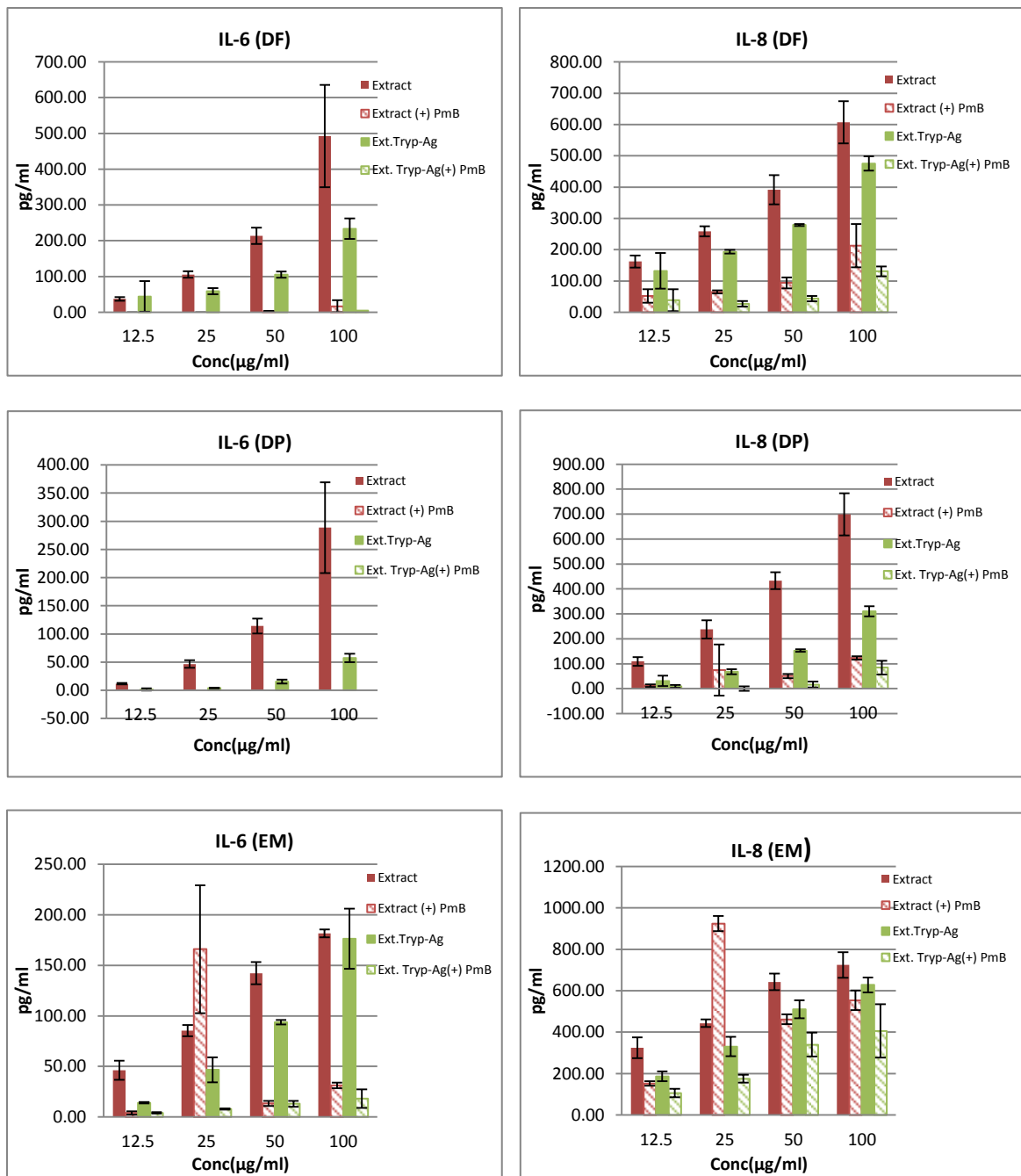


Figure 13 Modulatory effect of varying doses of trypsin agarose treated house dust mite extract on IL-6 and IL-8 cytokine secretion by normal dermal fibroblast (NHDF) cells. DF= *D. farinae*, DP= *D. pteronyssinus* and EM= *E. maynei*. Data are means \pm SD (n=3). Note, axes are different for each graph.

IV. Discussion

The three prevalent dust mite species in homes in temperate climates are *D. farinae*, *D. pteronyssinus* and *E. maynei* (Arlian et al., 2002). These house dust mite species produce allergens that are associated with allergic diseases such as asthma, perennial rhinitis and atopic dermatitis (Traidl-Hoffmann et al., 2009, Arlian and Platts-Mills, 2001). More than 21 different groups of house dust mite allergens have been characterized. These allergens are associated with mite body and fecal matter and include enzymes involved in the molting and digestive processes.

Aqueous extracts (vaccines) made from the bodies of these mites are used for diagnostic tests and immunotherapies. These extracts contain both allergen and non-allergen molecules. These allergenic and non-allergenic molecules can elicit innate (inflammatory) and adaptive immune responses in the skin, mucous membranes (eyes and nose) and the respiratory tract. These molecules can directly influence specific cell types in the tissues of these organs (Hammad et al., 2009, Trompette et al., 2009, Arlian et al., 2008). Thus, molecules from these mites can play an important role in the course of allergic and inflammatory reactions to them for both IgE mediated (atopic) and non-IgE mediated (non-atopic) reactions.

Previous studies have shown that whole house dust mite extracts (vaccines) or specific allergens (e.g. Der p 1) affect the function of many cell types, including lung epithelial cells, keratinocytes, fibroblasts and micro vascular endothelial cells of the skin, mast cells and basophils (Arlian and Morgan, 2011, Arlian et al., 2009, Hammad et al., 2009, Arlian et al., 2008, Kauffman et al., 2008). These effects include the modulation of cytokine and chemokine secretion, expression of cell adhesion molecules (CAMs), and cleavage of CD23, CD25 and CD40 of B-cells, T-cells and dendritic cells, respectively (Arlian et al., 2009, Arlian et al., 2008).

The entry of allergens can cause the onset of inflammation and immune responses (Vasilopoulos et al., 2007, Deleuran et al., 1998). Molecules in house dust mite extracts can degrade lung surface proteins and damage lung epithelium by disrupting intercellular tight junctions (Kauffman et al., 2009, Winton et al., 2008, Stewart et al., 1994 and Herbert et al., 1995). For example, group 1 allergens are cysteine proteases and have been shown to increase permeability in lung epithelium and also cause bronchial inflammation and the release of cytokines and chemokines involved in the inflammatory response (Winton et al., 2008, Stewart et al., 1994 and Herbert et al., 1995). House dust mite allergens have also been shown to affect skin barrier properties. House dust mite allergens that exhibit protease activity are capable of disrupting the

functioning of the epidermal barrier of the skin. Previous studies have also shown that whole house dust mite extract modulated the secretion of IL-6, IL-8, MCP-1 and M-CSF from dermal fibroblasts (Arlian and Morgan, 2011, Arlian et al., 2008). The purpose of this study was to determine what types of molecules and what type of mechanisms could be involved in modulating the secretion of these cytokines from adult normal human dermal fibroblast (NHDF) cells.

Specifically, in order to answer these questions, this study addressed the following goals:

- A. Determine the modulation of cytokine gene expression and cytokine secretion by NHDF cells in response to being challenged by whole dust mite extract
- B. Determine if low concentrations of whole dust mite extract and the time used to challenge the cells influenced IL-6, IL-8, MCP-1 and M-CSF cytokine secretion.
- C. Determine the role of endotoxin (LPS) in eliciting cell responses by a pathway involving Toll-Like Receptors (TLRs)
- D. Determine if proteins in the extracts were responsible for modulating cell function.

A. Modulation of cytokine gene expression and cytokine secretion by NHDF cells in response to being challenged by whole dust mite extract

Gene expression studies (figures 1 to 4) were done on NHDF cells that were challenged for 6, 12 and 24 hours with 50 µg/ml of whole dust mite extract. This assay was carried out to determine if there was a correlation between cytokine gene expression and actual cytokine secretion. This experiment also helped determine if induced gene expression was constant or if it occurred at the specific time intervals (on-off).

Arlian et al. (2008) and Arlian and Morgan (2011) demonstrated that cytokine release by NHDF cells and human skin equivalents that were challenged with *D. farinae* whole mite extract secreted high levels of IL-6, IL-8, MCP-1 and M-CSF when compared to the media control. Our current study showed that gene expression of NHDF cells challenged with 50 µg/ml *D. farinae* dust mite extracts for 6, 12 and 24 hours also induced cytokine gene expression for the same cytokines. Therefore these results suggest that cytokine secretion previously observed by Arlian et al (2008) was more likely a result of cytokine gene expression and not due to the release of stored cytokines in response to being challenged by *D. farinae* extract. When *D. farinae* extract was boiled, to denature the proteins in the extract, cytokine gene expression was no longer induced. These data suggest that cytokine gene expression and release of cytokines by NHDF cells challenged

with *D. farinae* whole dust mite extract was mainly influenced by protein molecules in this extract.

Arlian et al. (2008) and Arlian and Morgan (2011) demonstrated that *D. pteronyssinus* whole mite extract induced high levels of IL-8 and M-CSF secretion by NHDF cells and human skin equivalents and no change in secretion of IL-6 and MCP-1 when compared to the media control. The gene expression data from this study showed that extracts of *D. pteronyssinus* whole dust mite did not induce IL-6, IL-8, MCP-1 and M-CSF cytokine gene expression at 6, 12 and 24 hours. One possible explanation for IL-8 and MCP-1 cytokine secretion not corresponding with gene expression is that NHDF cells released stored cytokines in response to being challenged with *D. pteronyssinus* extract but the cells did not synthesize additional cytokines. Therefore, it appears that molecules in the *D. pteronyssinus* extract, unlike *D. farinae*, did not affect cytokine gene expression for IL-8 and M-CSF. This clearly demonstrates that the composition of the two mite extracts is very different and that they can have different effects on inflammation and the allergic reaction.

Like *D. farinae* and *D. pteronyssinus* NHDF cells challenged with *E. maynei* whole mite extract also secreted high levels of IL-8. Like *D. pteronyssinus*, IL-6, MCP-1 and M-CSF were comparable to media control for *E. maynei* stimulated cells (Arlian et al., 2008).

However, *E. maynei* whole dust mite extracts did not induce IL-6, IL-8, MCP-1 or M-CSF cytokine gene expression. One possible explanation for the difference in IL-8 cytokine secretion and gene expression is that NHDF cells respond to *E. maynei* whole mite extract by secreting stored IL-8, therefore not requiring IL-8 gene expression to occur at specific time intervals.

B. Determine if low concentrations of whole dust mite extract and the time used to challenge the cells influence cytokine secretion.

Normal human dermal fibroblasts (NHDF) were challenged with 5, 10 and 20 µg/ml of extract in the absence or presence of 50 ng/ml of LPS (figures 6 to 8). Our data showed that each dust mite extract influenced IL-6, IL-8, MCP-1 and M-CSF cytokine secretion differently. Molecules in *D. farinae* and *E. maynei* extract modulated cytokine secretion in a dose and time dependent manner. This effect was further enhanced when the cells were challenged with varying concentrations of *D. farinae* and *E. maynei* extracts that were spiked with LPS.

Different doses of *D. pteronyssinus* whole mite extract did not modulate cytokine secretion when various doses of extract were used to challenge NHDF cells. However when LPS was added to the various doses of extract, cytokine secretion was up regulated. This result demonstrated that doses of 5µg/ml, 10µg/ml and 20 µg/ml of *D.*

pteronyssinus did not induce cytokine secretion by NHDF cells. The data suggest that low concentrations of molecules in *D. pteronyssinus* extract, unlike *D. farinae* and *E. maynei*, were insufficient to induce cytokine secretion.

C. The role of endotoxins in eliciting immune responses by a pathway involving Toll-Like Receptors

Toll-like receptors (TLRs) are pattern recognition receptors that are capable of detecting pathogen-associated molecular patterns such as endotoxins. The TLR family comprises of several receptors that trigger immune responses. For instance, TLR-2 and TLR-4 recognize microbial components including lipopolysaccharide (LPS) which is a known endotoxin (Hammad et al., 2009, Cardoso et al., 2007, Takeda and Akira, 2005 and, Gangloff and Guenounou, 2004). TLR-2 and TLR-4 have also been shown to act through a pathway requiring MyD88 as an adaptor protein that binds the LPS molecule to the TLR to induce production of cytokines involved in the inflammation process (Takeda and Akira, 2005). Interestingly, the mite allergen Der p 2 can bind directly to the TLR and activate it without the MyD88 binding molecule.

House dust mite extracts that have been shown to affect lung epithelial and airway dendritic cells act through pathways that involve TLRs, specifically TLR-2 and TLR-4 (Hammad et al., 2009, Kauffman et al., 2006, Asokanthan et al., 2002). House dust mite

allergens from group 2 such as Der p 2 have been shown to prompt an immune response through TLR-4 (Trompette et al. 2009, Hammad et al., 2009). A recent study by Mueller et al. (2009) showed that Der p 7 like the group 2 allergens is able to interact with TLRs to promote an innate immune response.

The three house dust mite extracts used in this study contained different amounts of endotoxin. It is clear that the endotoxin in these extracts can affect fibroblast function. In order to determine how the different levels of endotoxin could affect cytokine secretion by NHDF cells, extracts were treated with polymyxin B (PmB) before the cells were challenged. PmB is an antibiotic that was used to neutralize endotoxins in the dust mite extracts. Thus, any cytokine secretion by NHDF cells that were challenged with house dust mite extract treated with PmB would be a result of non-endotoxin molecules in the extract.

When *D. farinae* and *D. pteronyssinus* extracts used to challenge cells were treated with PmB, NHDF cells no longer secreted IL-6 and IL-8 (figure 10). MCP-1 cytokine secretion was not affected. This suggests that endotoxins in *D. farinae* and *D. pteronyssinus* extracts influenced IL-6 and IL-8 cytokine secretion through a pathway involving TLRs. MCP-1 cytokine secretion by NHDF cells was not influenced by a pathway involving TLRs.

E. maynei extracts treated with PmB did not affect IL-6, IL-8 and MCP-1 cytokine secretion by NHDF cells (figure 10). Therefore, endotoxins in *E. maynei* extract did not influence IL-6, IL-8 or MCP-1 cytokine secretion. This result implies that cytokine secretion by NHDF cells could be result of allergens and other non-endotoxin molecules in *E. maynei* extract acting through TLR independent pathways.

D. The modulatory effect of proteins in the extract

Protease activated receptors (PARs) are G-protein coupled receptors that are activated by a cleavage of part of the receptor complex. PARs are activated by allergens that exhibit protease activity such as mite allergens that belong to groups 1 and 3 (Thomas et al., 2010, Kauffman et al., 2006 and Asokanathan et al., 2002).

In order to determine if allergens and other proteins that exhibit protease activity in the whole dust mite extracts affect cells through a pathway involving PARs, the extracts were boiled (5 or 30 minutes) or treated with trypsin agarose before the cells were challenged (figures 12, 13). Boiling the extract for 5 minutes would denature most of the heat labile proteins. Boiling the extract for 30 minutes would not only denature heat labile proteins but also affect endotoxin functions (Gao et al., 2009). Trypsin, a serine protease, was used to digest polypeptides at the carboxyl end of lysine or arginine

amino acids. This meant that polypeptides found in the extract would be digested into smaller peptide sequences. Both boiling and enzyme digestion could modify how the proteins in the extract affected cytokine secretion that was modulated by PARs activity.

1. Boiled extracts

NHDF cells that were challenged with *D. farinae*, *D. pteronyssinus* and *E. maynei* whole dust mite extracts boiled for 5 minutes, secreted similar amounts of IL-6 and IL-8 when compared to cells challenged with unboiled extract. These results imply that heat labile proteins in the extract are not essential in inducing the observed cytokine secretion by NHDF cells. This also suggests that other molecules or heat stable non protease proteins stimulated the cells via other receptors and not PARs.

In contrast, when NHDF cells were challenged with *D. farinae*, *D. pteronyssinus* and *E. maynei* extracts that were boiled for 30 minutes, IL-6 and IL-8 secretion decreased compared to cells that were challenged with unboiled extract. Therefore, boiling extracts for 30 minutes, unlike extracts boiled for 5 minutes appeared to denature all proteins, destroy LPS activity and inhibit stimulating properties of other molecules in the extracts. Thus, enzyme molecules in the extracts were no longer able to induce cytokine secretion by NHDF cells through pathways that involve PARs.

2. Trypsin agarose digested extracts

NHDF cells that were challenged with *D. farinae* and *D. pteronyssinus* trypsin agarose treated extracts showed a decrease in IL-6 and IL-8 cytokine secretion when compared to untreated extracts. This result implied that digesting proteins including allergens in the extract did not affect cytokine secretion by NHDF cells. Interestingly, NHDF cells that were challenged with *E. maynei* trypsin agarose treated extracts did not show decreased IL-6 or IL-8 secretion when compared to cells challenged with untreated extract. This result suggested that the protein regions required to activate PARs are unaffected by trypsin.

Modifying the extract by boiling for 5 or 30 minutes showed a different result than extracts digested with Trypsin. For instance extract boiled for 5 minutes was less effective at preventing IL-6 and IL-8 cytokine secretion than cells challenged with extract treated with trypsin agarose. A likely explanation for this result could be that trypsin agarose digested extracts contain fewer enzymes that could work through PARs than extracts boiled for 5 minutes. However extracts boiled for 30 minutes were more effective than boiling extract for 5 minutes or treating the extract with trypsin agarose at preventing IL-6 and IL-8 cytokine secretion by NHDF cells. A likely explanation for this result is that boiling the extract for a long time interval was sufficient in eliminating

more molecules in the extract that do not only affect cells through PARs but also through TLRs.

V. Summary

This study demonstrated that aqueous molecules in house dust mite extracts are capable of eliciting different cytokine responses by adult normal human dermal fibroblast (NHDF) cells. In some instances, cytokine gene expression and secretion were induced while in other instances only stored cytokine was released by NHDF cells. Extracts of the three different mite species contained different molecules that induced different responses. For instance *D. farinae* extract induced IL-6, IL-8 and MCP-1 cytokine gene expression at particular time intervals unlike *D. pteronyssinus* and *E. maynei*. Also, *D. pteronyssinus* extract induced cytokine secretion but not gene expression. The whole house dust mite extracts contain different concentrations of endotoxin (LPS) and different proteins. This study demonstrated that endotoxin molecules in *D. farinae* and *D. pteronyssinus* extracts played a major role in cytokine secretion. This study also confirmed that proteins in the extracts are capable of inducing cytokine secretion. Interestingly, it was also revealed that *E. maynei* whole extract contained molecules other than endotoxins and heat labile proteins that are able to elicit cytokine secretion by NHDF cells.

Information gained from this study as to how house dust mite extracts influence NHDF cells will be useful for understanding immune and inflammatory responses in the skin. This research will also help in the standardization and characterization of house dust mite extracts (vaccines) used for diagnostic and therapy purposes.

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